Platelet aggregation responses and virus isolation from platelets in calves experimentally infected with type I or type II bovine viral diarrhea virus

Paul H. Walz, Thomas G. Bell, Daniel L. Grooms, Lana Kaiser, Roger K. Maes, John C. Baker

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 examine for the presence of a virus-platelet interaction during BVDV infection. Colostrum-deprived Holstein calves were obtained immediately after birth, housed in isolation, and assigned to 1 of 4 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 by using adenosine diphosphate and platelet activating factor. 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, 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, but was not observed in calves infected with type I BVDV. Altered platelet function may be important as a difference in virulence between type I and type II BVDV infection.

Résumé

L'altération de la fonctionnalité des plaquettes est rapportée chez des veaux infectés expérimentalement avec le virus de la diarrhée virale bovine (BVDV). Afin d'évaluer en détail cette observation et d'examiner la présence d'une interaction plaquette-virus lors d'une infection par le BVDV, des veaux de race Holstein privés de colostrum obtenus immédiatement après leur naissance furent gardés en isolement et assignés à un des 4 groupes (un groupe témoin et 3 groupes traités). Les veaux témoins (n = 4) *furent inoculés à blanc, alors que les veaux des groupes infectés* (n = 4 *dans chaque groupe) reçurent* 10^7 $TCID_{50}$ *de BVDV 890 (type II), BVDV 7937 (type II) ou BVDV TGAN (type I) par instillation intra-nasale. Du sang complet fut prélevé avant (jour 0) et aux jours 4, 6, 8, 10 et 12 suivant l'inoculation afin de vérifier la fonction des plaquettes par mesure optique de l'agrégation en utilisant l'adénosine diphosphate et le facteur d'agrégation des plaquettes. Le pourcentage maximum d'agrégation et la pente de la courbe d'agrégation diminuèrent dans le temps chez les veaux infectés par le BVDV; toutefois, des différences significatives (ANOVA sur des mesures répétées de rangs de Freidman,* $P < 0,05$) *ne furent observées que chez des veaux infectés avec les isolats de BVDV de type II. Le virus du BVD ne fut pas isolé des veaux témoins, mais fut isolé de tous les veaux infectés avec les deux isolats de type II du VBVD à partir du jour 4 jusqu'au jour 12 après l'inoculation. Chez les veaux infectés avec le VBVD de type I le virus fut isolé à partir d'un des quatre veaux aux jours 4 et 12 après l'inoculation et de tous les veaux aux jours 6 et 8 après l'inoculation. Une altération de la fonction des plaquettes fut observée chez les veaux infectés avec les deux isolats de VBVD de type II, mais pas chez les veaux infectés avec le VBVD de type I. L'altération de la fonction des plaquettes et une association entre le virus et les plaquettes pourraient être important comme différence dans la virulence entre une infection par le VBVD de type I et celui de type II.*

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Introduction

Bovine viral diarrhea virus (BVDV), a member of the genus *Pestivirus* within the family *Flaviviridae*, is an economically important viral pathogen of cattle with a worldwide distribution (1). Isolates of BVDV can be classified into 2 biotypes, noncytopathic and cytopathic, and 2 genotypes, type I and type II (2). 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 (3). However, type II BVDV has been associated with outbreaks of severe peracute disease (4,5) and a hemorrhagic syndrome characterized by thrombocytopenia, bleeding, and death (6–8).

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 with qualitative platelet defects (thrombocytopathy), in which platelets have altered functional characteristics (9). Impairment of the hemostatic response may result in clinical signs of hemorrhage. Thrombocytopenia associated with type II BVDV infection has been reported in adult cattle and calves under natural conditions (7), and has been experimentally reproduced using different isolates of type II BVDV (8,10–14).

In addition to thrombocytopenia, altered platelet function has been reported following experimental type II BVDV infection in calves (15). Although the mechanism of platelet dysfunction was undetermined, BVDV was isolated from purified platelets during the observed platelet dysfunction, suggesting a virus-platelet interaction as a possible cause. However, a single type II BVDV isolate was examined in that study, and it is unknown if a virus-platelet interaction exists during infection with type I BVDV isolates or other type II BVDV isolates. In addition, it is unknown if other BVDV isolates are capable of altering platelet function. The purpose of the present study was to further evaluate the ability of BVDV isolates to alter platelet function and to further examine for the presence of a virus-platelet interaction during BVDV infection. 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.

Materials and methods

Animals

Colostrum-deprived newborn male Holstein calves (n = 16) were obtained immediately after birth from local dairy farms. The calves were moved to an isolation facility, allocated to 1 of 4 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 3 serial serum samples obtained prior to the collection of plasma. In addition,

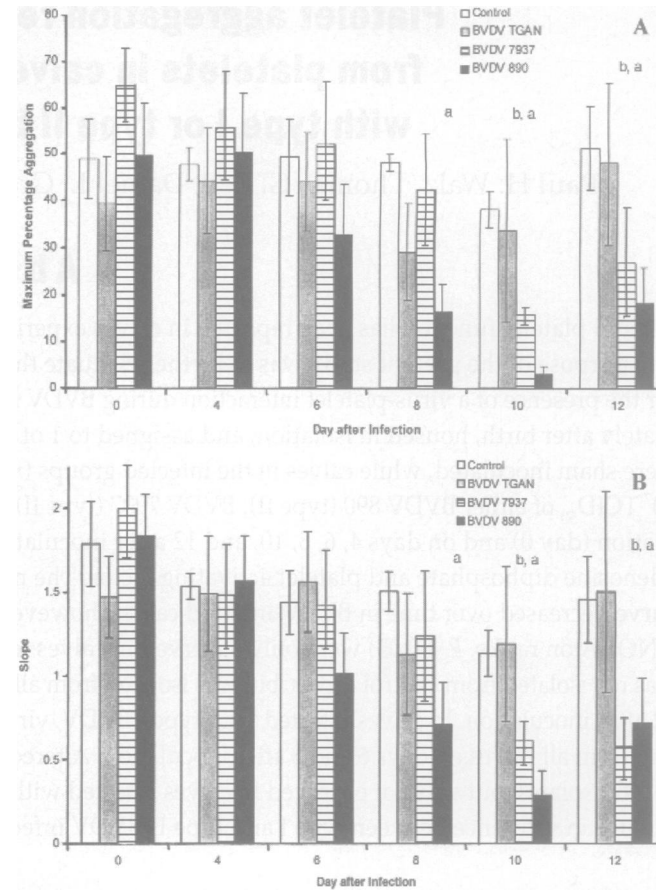

Figure 1. Platelet aggregation responses (A — maximum percent aggregation; B — slope of the aggregation curve) induced by adenosine diphosphate at a concentration of 100 μM for uninfected control calves, calves infected with the type I isolate BVDV TGAN, calves infected with the type II isolate BVDV 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: a = BVDV 890 post-inoculation vs. pre-inoculation (day 0); b = BVDV 7937 post-inoculation vs. pre-inoculation (day 0).

calves were given injections of vitamins A and D (Butler Company, Dublin, Ohio, USA) and vitamin E and selenium (Schering-Plough Animal Health, Kenilworth, New Jersey, USA), as well as a commercial oral monoclonal antibody preparation against *E. coli* K99 pilus antigen (Schering-Plough Animal Health). Calves were fed a non-medicated milk replacer, according to label directions. 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 3 noncytopathic BVDV isolates were used for the experimental infection of calves: BVDV 890 (Dr. Steve Bolin, National Animal Disease Center, Ames, Iowa, USA), BVDV 7937 (Dr. Clayton Kelling, University of Nebraska, Lincoln, Nebraska, USA), and BVDV TGAN (Dr. Steve Bolin, National Animal Disease Center). The BVDV 890 is a type II isolate that has been previously shown to induce thrombocytopenia and alter platelet function in experimentally infected calves (8,12,15). The BVDV 7937 (type II)

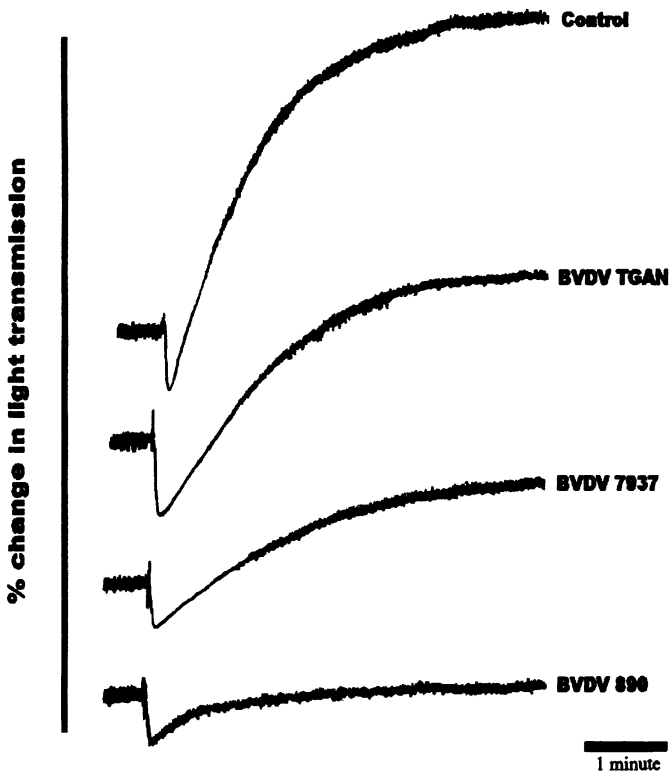

Figure 2. Aggregometer tracings of bovine platelet rich plasma suspensions stimulated with adenosine diphosphate at a concentration of 100 μM. The 4 tracings represent the aggregation response curves of bovine platelets obtained from a calf in each group on day 10 post-inoculation. In the BVDV-infected calves, the lower percent change in light transmission, compared to the other aggregation tracing from the control calf, represents a depressed aggregation response.

and BVDV TGAN (type I) are isolates that in previous reports did not induce thrombocytopenia (8,16); however, these isolates have not been evaluated for their ability to alter platelet function. The viral isolates were propagated in bovine turbinate cells in Eagle's minimum essential medium (EMEM; JRH Biosciences, Lenexa, Kansas, USA) containing 10% fetal equine serum (FES; Sigma Chemical Company, St. Louis, Missouri, USA), L-glutamine (Gibco BRL, Life Technologies, Grand Island, New York, USA), penicillin G (Sigma Chemical Company), and streptomycin (Sigma Chemical Company). The viral titer of the inoculum was determined according to previously described methods (17).

After a 3-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 (before 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-enriched plasma was obtained by 2 centrifugation steps at 1324 × g for 60 s and 30 s, with the platelet-enriched plasma being removed after each centrifugation. Platelet-depleted plasma was obtained by centrifugation of the remaining blood at 1324 × g for 13 min. Platelets were counted manually using a white blood cell microcollection system (Becton Dickinson, Franklin, New Jersey, USA) and a hemocytometer.

Platelet aggregation responses

Aggregation studies were performed using optical aggregometry (Chronolog Corporation, Havertown, Pennsylvania, USA). Aggregation agonists used for this study were adenosine diphosphate (ADP; Sigma Chemical Company), at concentrations of 10 and 100 μM, and platelet activating factor (PAF; Calbiochem Biochemicals, San Diego, California, USA), at concentrations of 0.1 and 1.0 μM. Platelet-enriched plasma was adjusted to 300 000 platelets/μL with homologous platelet-depleted plasma. Adjusted platelet-enriched plasma suspensions were then divided into 0.5-mL aliquots in glass cuvettes and held at room temperature for 30 min. The platelet-enriched plasma suspensions were warmed for 5 min 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 platelet-enriched plasma to establish the 0% aggregation limit and platelet-depleted plasma to establish the 100% aggregation limit. The sample was allowed to stir at 900 rpm for approximately 30 s, 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 manually calculated for each aggregation run, according to previously described methods (18). All aggregation studies were done at least in duplicate, and the mean value was obtained. All studies were completed within 5 h of collection of blood samples.

Virus isolation from platelets

Platelets were gel-filtered and washed to remove plasma constituents. Platelet enriched plasma was collected as described above and 1 μL of 1 μM prostaglandin E_1 (Sigma Chemical Company) was added per 1 mL platelet enriched plasma prior to centrifugation at 800 × g for 15 min. Plasma supernatant was extracted 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, 5.5 mM dextrose, pH 7.4). The platelet suspension was transferred to a 10 mL polystyrene column containing Sepharose 4B (Sigma Chemical Company). The eluate was collected, washed three times in 10 mL of HBSS, resuspended in HBSS, and counted manually. Ten million (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 d 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 d 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 (19).

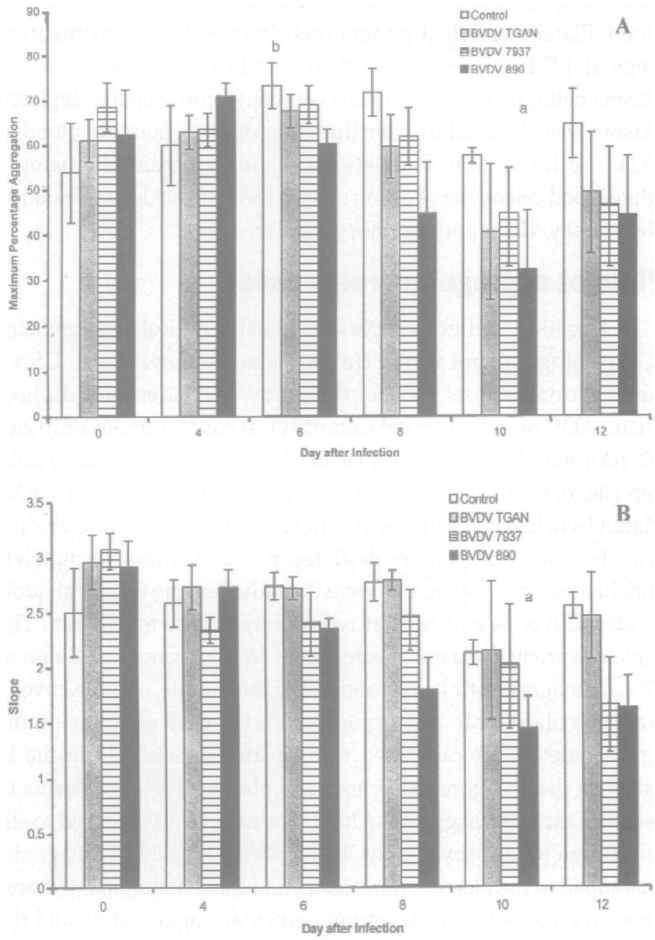

Figure 3. Platelet aggregation responses (A — maximum percentage aggregation; B — slope of the aggregation curve) induced by platelet activating factor at a concentration of 0.1 μM for uninfected control calves, calves infected with the type I isolate BVDV TGAN, calves infected with the type II isolate 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: a = BVDV 890 post-inoculation vs. pre-inoculation (day 0); b = Control post-sham inoculation vs. pre-sham inoculation (day 0).

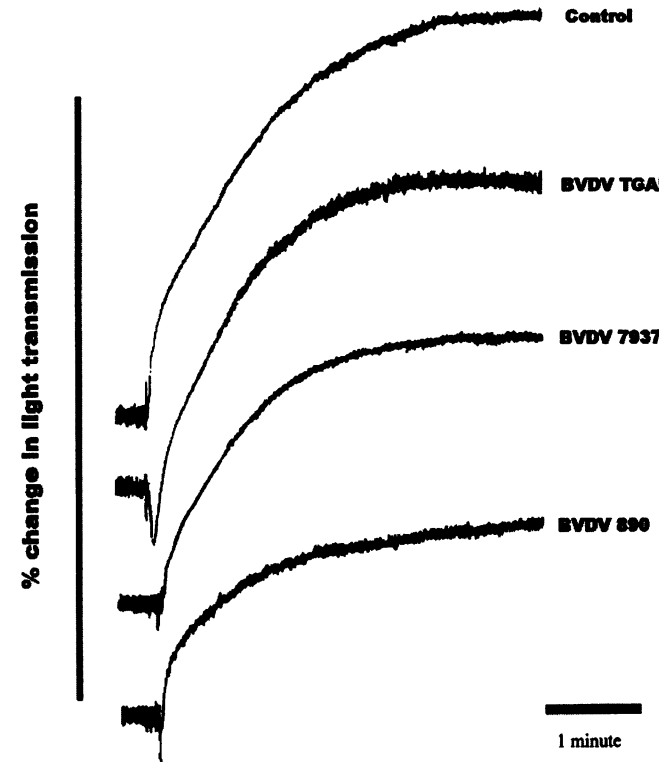

Figure 4. Aggregometer tracings of bovine platelet-rich plasma suspensions stimulated with platelet activating factor at a concentration of 0.1 μM. The 4 tracings represent the aggregation response curves of bovine platelets obtained from a calf in each group on day 10 post-inoculation. In the BVDV-infected calves, the lower percent change in light transmission, compared to the other aggregation tracing from the control calf, represents a depressed aggregation response.

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 (before inoculation) to days 4, 6, 8, 10, and 12 sample periods (after inoculation) by using Dunn's multiple comparisons test.

Results

Aggregation responses with adenosine diphosphate (ADP)

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 2 groups of calves inoculated with type II BVDV, with the greatest depression occurring in the calves inoculated with the type II isolate BVDV 890. Zero aggregation, which is defined as a failure to record an increase in light transmission above the 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. Statistically significant differences in the maximum percentage aggregation and slope of the aggregation curve were observed from post-inoculation days 6 through 12 in BVDV 890-infected calves. In calves infected with BVDV 7937, statistically significant differences were observed from post-inoculation days 8 through 12 in the maximum percentage aggregation and on post-inoculation days 8 and 10 in the slope of the aggregation curve. In calves infected with the type I isolate BVDV TGAN, a statistically significant difference in platelet function was not observed between pre- and post-inoculation samples in response to 10 μM ADP.

In 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 aggregation in the BVDV-infected calves, but a decrease in the maximum percentage aggregation and slope was only observed in calves infected with type II BVDV (Figure 1). 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

Table I. Virus isolation results for purified platelet samples from calves experimentally infected with type I or type II bovine viral diarrhea virus (BVDV)[a]

Day after infection	Control (uninfected)	BVDV TGAN (type I BVDV)	BVDV 7937 (type II BVDV)	BVDV 890 (type II BVDV)
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] numerator: number of calves that were virus-positive; denominator: number of calves in group

Figure 2. Zero aggregation, as previously defined, was observed in response to 100 μM ADP in 2 of 4 calves infected with the type II isolate BVDV 890 on day 10 after inoculation.

Aggregation responses to platelet activating factor (PAF)

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 (Figure 3). As similar to ADP 100 μM, 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. A statistically significant difference was not detected between pre- and post-inoculation samples in calves infected with the type I isolate BVDV TGAN.

In response to 1.0 μM PAF, maximum percentage aggregation and slope in calves infected with the type II BVDV 890 were significantly depressed on day 10 and days 8, 10, and 12 after inoculation, respectively, when compared to day 0. In calves infected the type II isolate BVDV 7937, statistically significant differences were not observed with respect to the maximum percentage aggregation, but were observed in the slope of the aggregation curve at post-inoculation days 10 and 12. A statistically significant difference was not detected between pre- and post-inoculation samples in calves infected with either the type II isolate BVDV 7937 or the type I isolate BVDV TGAN.

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 type II BVDV. Virus was also isolated from all calves infected with the type I BVDV TGAN, but was not isolated from all the calves at all post-inoculation time periods (Table I).

Discussion

Platelets are essential for normal hemostasis, and alterations in their function may lead to hemorrhage (9). Depression of platelet function has been previously demonstrated in calves experimentally infected with the type II isolate BVDV 890 (15), 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 (12). 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 (15), the mean values for maximum percentage aggregation and slope 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 (16), 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 capable at depressing platelet function than type II BVDV isolates which 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 (8). 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 (20). 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.

Although the exact mechanism responsible for the diminished platelet response in BVDV- infected calves is unknown, a direct interaction between BVDV and platelets is not likely the mechanism. In support of this statement, platelet dysfunction was not observed in calves inoculated with the type I isolate BVDV TGAN, and yet we were able to demonstrate virus association with platelets in all calves within the group. The association of BVDV with platelets has been demonstrated in previous studies using virus isolation procedures (8) and immunofluorescent antibody testing (6,11). Platelets are small, anucleate, cytoplasmic fragments that are derived from precursor cells in the bone marrow, the megakaryocytes (21). Bovine viral diarrhea virus infection of megakaryocytes has also been demonstrated through immunohistochemical and immunofluorescent antibody techniques identifying BVDV antigen (6,16,22,23). 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 older platelets in the peripheral circulation. As platelets age in the peripheral circulation, they become less sensitive to aggregatory stimuli (24,25). In contrast, younger platelets are significantly more sensitive than aged platelets to the aggregation agonists, ADP and thrombin (25). 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.

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 10 μM, as the calves aged. A similar depression in platelet function in response to ADP 10 μM was observed in an earlier study as well (15). 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 d of age was also observed in a previous study (12). Thus, age-matched control calves are important in studying BVDV infection and platelet disorders in neonatal calves. Another observation was 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 pre-inoculation sample period.

In summary, we have demonstrated altered platelet function using 2 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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