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FEBRILE RESPONSE AND DECREASE IN CIRCULATING LYMPHOCYTES FOLLOWING ACUTE INFECTION OF WHITE-TAILED DEER FAWNS WITH EITHER A BVDV1 OR A BVDV2 STRAIN

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ABSTRACT: Although commonly associated with infection in cattle, bovine viral diarrhea viruses (BVDV) also replicate in many domestic and wildlife species, including cervids. Bovine viral diarrhea viruses have been isolated from a number of cervids, including mule deer (*Odocoileus hemionus*), European roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*), white-tailed deer (*Odocoileus virginianus*), and mouse deer (*Tragulus javanicus*), but little information is available regarding clinical presentation and progression of infection in these species. In preliminary studies of experimental infection of deer with BVDV, researchers noted seroconversion but no clinical signs. In this study, we infected white-tailed deer fawns that were negative for BVDV and for antibodies against BVDV, with either a type 1 or a type 2 BVDV that had been isolated from white-tailed deer. Fawns were monitored for changes in basal temperature, circulating lymphocytes, and platelets. The clinical progression following inoculation in these fawns was similar to that seen with BVDV infections in cattle and included fever and depletion of circulating lymphocytes. Because free-ranging cervid populations are frequently in contact with domestic cattle in the United States, possible transfer of BVDV between cattle and cervids has significant implications for proposed BVDV control programs.

Key words: Acute infection, bovine viral diarrhea virus, deer *Odocoileus virginianus*.

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is an umbrella term for two species of viruses, BVDV1 and BVDV2 (*Pestivirus*, *Flaviviridae*; Thiel, et al. 2005). Bovine viral diarrhea viruses are found worldwide, and acute infections in cattle result in enteric, respiratory, and reproductive diseases of varying severity, depending on the BVDV strain, the immune and reproductive status of the host, and the presence of secondary pathogens. Although most commonly associated with cattle, there is evidence based on virus isolation and serology that BVDV replicates in a wide variety of domesticated and wild ruminants, including cervids such as white-tailed deer (*Odocoileus virginianus*), mule deer (*Odocoileus hemionus*), fallow deer (*Dama dama*), elk (*Cervus elaphus*), European roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*), and mouse deer (*Tragulus javanicus*) (Couvillion et al.,

1980; Frolich, 1995; Fischer et al., 1998; Frolich and Flach, 1998; Cuteri et al., 1999; Tessaro et al., 1999; Nielsen et al., 2000; Van Campen et al., 2001; Grondahl et al., 2003; Uttenthal et al., 2005). Acute BVDV infections in cattle are accompanied by immune suppression due, at least in part, to the death of immune cells within lymph nodes and gut associated lymphoid tissue and reduction in numbers of circulating white blood cells. The suppression of the immune system leaves infected cattle vulnerable to secondary infections (Brackenbury et al., 2003).

Because free-ranging cervid populations are frequently in contact with domestic cattle in the United States, possible transfer of BVDV between cattle and cervids has significant implications for proposed BVDV control programs. Previous studies of experimental infection of deer and elk with BVDV have noted seroconversion, viremia, and viral shedding but no clinical signs (Van Campen et

al., 1997; Tessaro et al., 1999). In those studies, the viral strains used were derived from bovine BVDV cases. Further, the immunologic background of the cervids used in those studies was not fully characterized. Those studies used cervids that tested serologically negative for BVDV under the assumption that serologically negative equated with immunologically naive. However, we have observed in studies with cattle that exposure to BVDV when colostral antibodies are present results in a protective T cell response that is not accompanied by a serologic response (Ridpath et al., 2003; Endsley et al., 2004). The objective of this study was to investigate the clinical presentation in white-tailed deer, with known BVDV exposure, following inoculation with a BVDV that had been previously isolated from white-tailed deer.

MATERIALS AND METHODS

Virus isolation, characterization, and propagation

The two viruses, R03-24272 and R03-20663, used in this study were isolated from two different white-tailed deer carcasses submitted to South Dakota State University for testing. Viruses were propagated in the Maden Darby bovine kidney cell line (MDBK) and characterized by phylogenetic analysis of 5' untranslated region (UTR) sequences as described previously (Ridpath et al., 2006). Cultured cells were grown in McCoy's medium supplemented with 10% fetal bovine serum tested free of BVDV and antibodies against BVDV.

Monoclonal antibody (MAb) binding was determined, using MAbs that recognized the E2 region as described (Ridpath et al., 1994). Both viruses belonged to the noncytopathic biotype as determined by lack of cytopathic effect in cultured MDBK cells.

Handling and treatment of deer complied with the Animal Welfare Act as Amended (7 USC, 2131–2156). Two to 4-wk-old bottle-fed white-tailed deer fawns were purchased from two commercial breeders and tested free of antibodies against BVDV by virus neutralization test and free of BVDV by virus isolation from buffy coat (BC). These fawns were purchased from commercial herds that were negative for BVDV exposure as determined by screening of pooled sera for antibodies against

BVDV1a, BVDV1b, and BVDV2 strains. Deer were cohoused by experimental group in climate-controlled Biosafety Laboratory 2 (BL2) barns and bottle-fed goat milk for the duration of the experiment.

Fawns were divided into three groups as follows: noninoculated control ($n=2$), inoculated with BVDV1b strain ($n=4$), and inoculated with BVDV2 strain ($n=4$). Inoculated fawns received 3.5 ml of 5.6×10^6 tissue culture infectious dose (TCID)₅₀/ml by the oral/nasal route. This inoculation dose was selected because earlier studies of clinical presentation and pathogenesis accompanying BVDV infections in cattle used similar doses (Bolin and Ridpath, 1992; Liebler-Tenorio et al., 2002, 2003), thus allowing comparison with studies in bovines. Basal temperatures were recorded daily, and blood samples were collected before inoculation and on days 3, 6, 9, 11, and 13 postinoculation for blood count (Vacutainer[®] Buffered sodium citrate. [9:10] 0.129 M 3.8%, BD Diagnostics, Franklin Lake, New Jersey, USA), serology (Vacutainer[®] SST Gel and Clot Activator tubes), and virus isolation (Vacutainer[®] sodium heparin tubes). In addition, serum samples were collected 30 days after inoculation.

Lymphocyte and platelet counts were determined using a cytometer (Hemavet 1500) per the manufacturer's directions (CDC Technologies, Inc., Oxford, Connecticut, USA). Cytometer settings were normalized for deer samples by the manufacturer. Viral neutralizing titers in serum were determined using the viral strains 296c and TGAC as reference strains as described (Bolin and Ridpath, 1990). For viral isolation, the BC was separated by centrifugation ($800 \times G$, 20 min) and put through one freeze/thaw cycle (-20 C/ 25 C). A 0.5-ml aliquot of BC freeze-thaw lysate was mixed with 0.5 ml of media. The resulting mixture was used to inoculate a 25-cm^3 , 60–70% confluent, flask of MDBK cells. After rocking at 37 C for 1 hr, the inoculum was removed from the cells and replaced with 5 ml of cell culture media (McCoy's cell culture medium supplemented with 10% fetal bovine serum). After 5 days, the cell culture (including media) was frozen at -80 C. After thawing to 25 C, 1 ml of the resulting lysate was added to a fresh 25-cm^3 flask of MDBK cells. After rocking for 1 hr at 37 C, 4 ml of cell culture medium was added. After incubating for 5 days, total RNA was prepared from the culture and tested for BVDV as described (Ridpath et al., 2006).

Statistical analysis was done to determine whether the variation from baseline values (expressed as a percentage of baseline) dif-

ferred between the experimental groups (animals exposed to virus) and the control group (animals not exposed to virus). The test performed was a two sample *t*-test confidence interval with the assumption that the standard deviations of the samples were not equal (Snedecor and Cochran 1989).

As samples were collected on five dates postinoculation and two different viruses were used, 10 hypothesis tests were performed (two tests for each of five sampling dates). The hypothesis test was the following:

$$H_0 : \mu_{exp} - \mu_{control} = 0$$

$$H_1 : \mu_{exp} - \mu_{control} \neq 0$$

where $\mu_{control}$ represents the mean percentage of baseline for the control group, and μ_{exp} represents the mean percentage of baseline one of an experimental group. This hypothesis test was done for each day and each experimental group.

The confidence interval formula was as follows:

$$(x_{exp} - x_{control}) \pm t\left(\alpha/2, n_{exp} + n_{control} - 2\right) \\ \times S_p \sqrt{(1/n_{exp} + 1/n_{control})}$$

where $S_p = \{[(n_{exp}-1)S_{exp}^2 + (n_{control}-1)S_{control}^2] / (n_{exp} + n_{control} - 2)\}^{1/2}$, x_{exp} = sample mean percentage change for experimental group, $x_{control}$ = sample mean percentage change for control group, s_{exp} = sample standard deviation percentage change for experimental group, $s_{control}$ = sample standard deviation percentage change for control group, n_{exp} = number of animals in experimental group, $n_{control}$ = number of animals in control group, and $t(\alpha/2, n_{exp} + n_{control} - 2)$ represents the *t*-critical value from the Student's normal distribution at the α level of significance with $n_{exp} + n_{control} - 2$ degrees of freedom.

The assumed level of significance was $\alpha = 0.05$.

RESULTS

Based on phylogenetic analysis of sequences from the 5' UTR region, one virus (R03-24272) was determined to be a BVDV1b strain and the other virus (R03-20663) was determined to be a BVDV2 strain. Although phylogenetic analysis did not show these viruses to be greatly divergent from type strains of BVDV,

MAb-binding panels revealed unique binding-panel results for R03-24272 (Table 1), which suggests an antigenic divergence in the E2 region. Intriguingly, the same panel results were observed for a BVDV1b strain that was isolated from swine. R03-24272, R03-20663, and the BVDV1b strain isolated from swine replicated to titers in bovine cells that were similar to BVDV isolated from bovines (data not shown). The BVDV isolated from swine replicated to equivalent titers in porcine and bovine cell lines. Because a white-tailed deer cell line is not available, it was not possible to compare replication in deer and bovine cell lines from the viruses isolated from deer.

Basal temperatures were recorded from days -3 to +21 following inoculation. The average temperature of all fawns between days -3 to -1 was 39.2 C (102.0 F). At day 0, no fawn had a recorded temperature >39.2 C. Further, recorded temperatures for control animals never exceeded 39.2 C (Table 2). In addition, one fawn (3851), inoculated with R03-24272, never had a recorded temperature greater than 39.2 C. In contrast, seven of the eight other fawns exposed to virus had recorded temperatures above 39.2 C ranging up to 40.4 C for 3-10 days. Of these seven fawns, one exhibited its first recorded temperature above 39.2 C on day 1, five on day 2, and one on day 3.

None of the fawns at baseline (day -2 inoculation) had an antibody titer greater than 4 against a reference BVDV1b strain (TGAC) or a reference BVDV2 strain (296c), and no serum collected from control animals, at any time point, had a titer of >4. In contrast, three of four animals infected with R03-24272 and two of four animals infected with R03-20663 had titers greater >4 by day 21 postinoculation. Viral titers were higher against a virus from the same genotype as the inoculation virus. That is, fawns exposed to R03-24272, which is a BVDV1b strain, had a mean titer of 4.9 (log base 2) against TGAC, another BVDV1b strain,

TABLE 1. Monoclonal antibody (MAb) binding panel.^a

	BVDV 1b lab strains		BVDV 1b field strains				BVDV1b strains from deer	
	NY-1	TGAN	Gill93	Hess9804	NMSU925	Hess135-89	(R03-20633)	Pig
CA-82	Y	Y	Y	Y	Y	Y	N	N
CA-36	Y	N	N	Y	Y	Y	N	N
CA-34	Y	Y	Y	Y	Y	Y	Y	Y
CA-3	Y	Y	Y	Y	Y	Y	N	N
CA-1	Y	Y	Y	Y	Y	Y	N	N

^a MAb binding panels were derived by immunoperoxidase staining of infected cell monolayers using MAbs specific for the E2 structural protein of bovine viral diarrhoea viruses (BVDV). All strains used belonged to the BVDV1b subgenotype. Strains NY-1 and TGAN are standard laboratory strains. Strains Gill93, Hess9804, NMSU925, and Hess135-9 were all isolated from persistently infected cattle identified between January 2005 and January 2006. The deer isolate, R03-20633, is described in this paper. The strain Pig was isolated from porcine tissues in 2005.

compared with a mean titer of 2.1 against 296c, a BVDV2 strain. Similarly, fawns exposed to R03-2633, which is a BVDV2 strain, had a mean titer of 3.1 (log base 2) against 296c, compared with a mean titer of 1.4 against TGAC.

Virus was not isolated from BC samples collected from any of the fawns at the baseline bleed or after day 9. Virus was not isolated from either of the two control animals on any sampling date. Between day 3 and day 9 postinoculation, virus was isolated from BC samples collected from two of four fawns infected with R03-24272 and three of four fawns infected with R03-20633 (Table 2). A region of the 5' UTR

was amplified and sequenced from viruses isolated from BC samples. Comparison of these sequences confirmed that the isolated virus originated from the inoculation strain. Combining serology and virus isolation data demonstrates that all eight fawns exposed to virus became infected.

One of the fawns (3855), inoculated with R03-24272, displayed lethargy and an unsteady gait between day 4 and day 8. This fawn also developed the highest temperature of 40.4 C and the second-longest running elevated temperature (>39.2 C for 10 days). Another fawn (3858) inoculated with R03-24272 developed a persistent cough observed between

TABLE 2. Temperature, virus isolation, and seroconversion in white-tailed fawns acutely infected with one of two BVDV strains.

	Days temperature between 39.2 C and 39.4 C	Days temperature between 39.3 C and 40.0 C	Days temperature greater than 40.0 C	Seroconversion Day 21	Virus isolation
Noninfected					
Deer 17	0	0	0	—	—
Deer 127	0	0	0	—	—
R0-24272 infected					
Deer 3855	3	7	2	+	—
Deer 3858	0	6	0	—	+
Deer 3851	0	0	0	+	—
Deer 6	2	3	0	+	+
R0-20633 infected					
Deer 3856	2	1	0	+	+
Deer 3868	0	8	0	—	+
Deer 4	2	3	0	—	+
Deer 5	2	10	0	+	—

TABLE 3. Average lymphocyte values as proportion of baseline values.

Day post inoculation	20633 ^a	24272 ^a	Control
Day 3	0.43 ^b	0.47 ^b	1.05
Day 6	0.37 ^b	0.57 ^b	0.97
Day 9	0.57	0.60	0.80
Day 11	0.85	1.205	0.98
Day 13	0.66	1.146	0.96

^a Virus used to inoculate animals in group.

^b Value significantly different from control.

day 10 and day 13. All fawns inoculated with virus had reduced circulating lymphocyte levels compared with baseline values. This reduction was most pronounced on days 3 and 6 (Table 3) and averaged greater than 60% in animals infected with R03-20633 and greater than 50% in animals infected with R03-24272. Because there was variation in the baseline circulating lymphocyte values among the 10 animals in the study, values, for statistical analysis, were normalized by expressing circulating lymphocyte levels as a proportion of the baseline level (Table 3). The closer the proportion value is to 1, the less the change from baseline levels, whereas proportion values less than 1 reflect a decrease from baseline counts and proportion values greater than 1 reflect an increase. For both experimental groups, a statistically significant reduction in circulating lymphocytes, as compared with the control group, was observed on days 3 and 6 (Table 3). The confidence intervals calculated for average circulating lymphocyte values for animals infected with 20633 versus control animals on days 3 and 6 were -1.1379 to -0.0961 and -1.1122 to -0.0957 , respectively. Similarly, the confidence intervals for animals infected with 24272 compared with control animals on days 3 and 6 were -0.9077 to -0.2552 and -0.7732 to -0.0325 , respectively. A statistically significant difference from the control group, in circulating lymphocytes, was not observed for either experimental group on days 9, 11,

or 13. No statistically significant difference in platelet counts was observed between experimental and control groups on any testing date (data not shown).

DISCUSSION

In cattle, infection with BVDV strains typically results in biphasic pyrexia and a decrease in circulating lymphocytes (Evermann and Barrington, 2005). Viremia in cattle usually occurs between day 3 and day 11 postinoculation with a lymphocyte drop that is most pronounced between day 3 and day 6 (Evermann and Barrington, 2005). This study revealed pronounced decreases in circulating lymphocytes in all fawns exposed to either a BVDV1b or a BVDV2 strain. An elevation in basal temperature was also observed in seven of eight fawns. The largest decrease in circulating lymphocytes occurred on days 3 and 6 and viremia occurred between day 3 and day 9. Thus, the course of experimental infection observed in fawns in this study was very similar to that reported for experimental infection in cattle using similar virus dosages (Bolin and Ridpath, 1992; Liebler-Tenorio, 2002, 2003). In cattle, it is assumed that the lymphocyte drop is associated with the immune suppression commonly observed with BVDV infections. Further research needs to be done to confirm whether immune suppression also occurs in cervids.

Why were clinical signs observed in this study but not in previous studies? That answer may lie in the three ways this study differed from those previous studies: 1) the viruses used to inoculate deer were isolated from deer in the field, rather than using BVDV strains that had been isolated from cattle; 2) the immunologic background of these fawns was more fully characterized; and 3) bottle-fed fawns were used. Bottle-fed fawns are more amenable to human handling. In a wildlife species, such as white-tailed deer, the fight-or-flight response elicited by the

presence of caretakers may mask clinical signs, such as depression, and skew blood counts. Bottle-fed fawns are less likely to have a "bolt" response to the caretakers that feed and handle them multiple times a day. The acclimation of the fawns to human handling results in blood counts, basal temperature determinations, and observations of behavior that are more reliable.

Because the acute infection appears so similar between cervids and cattle, the next logical question is whether cervids develop persistent infections similar to cattle. The experimental infections observed in this study did not result in a clinical disease so severe it would endanger a pregnancy, thus the two viral strains used in these studies would be good candidates for fetal infection studies. Experiments are now underway to determine whether exposure of pregnant deer to these two BVDV isolates results in the birth of persistently infected fawns.

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