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OBSERVATIONS ON BLUETONGUE AND EPIZOOTIC HEMORRHAGIC DISEASE VIRUSES IN WHITE-TAILED DEER: (1) DISTRIBUTION OF VIRUS IN THE BLOOD (2) CROSS-CHALLENGE[1]

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Abstract: The viruses of bluetongue and epizootic hemorrhagic disease produced cell associated viremias in white-tailed deer *(Odocoileus virginianus).* Highest virus titers were associated with the erythrocytes. The titers of virus in the erythrocyte fraction of blood were consistently higher than those in the leukocyte fraction, although virus persisted in both fractions for approximately the same length of time. All detectable viremia disappeared within 6 to 8 days following the development of virus-specific neutralizing antibodies. These antibodies failed to confer protection against challenge with virulent heterologous virus, although the time of death was delayed 3 to 11 days in comparison with control deer.

INTRODUCTION

Results of studies relating to the morphological, physiochemical properties[1,11,17] and to the epizootiological, clinical and pathological patterns[23] of bluetongue virus (BTV) and of epizootic hemorrhagic disease virus (EHDV), have suggested that these two agents are very closely related if not the same virus. Serologically, however, EHDV has been shown to cross-react with type 6 BTV strains but not with members of the type 10 BTV group.[11,16] An important consideration of possible relationships between BTV and EHDV would be their ability to cross-protect. Results to date, however, are limited and inconsistent.

Elk *(Cervus canadensis)* when experimentally infected with either BTV or EHDV respond with an inapparent disease and specific immunity.[3,12] When BTV immune elk were subsequently challenged with virulent EHDV, they survived and had an "amnestic response" to BTV as measured by agar gel diffusion.[12]

Vosdingh et al.[20] reported that white-tailed deer *(Odocoileus virginianus)* which were immune to EHDV withstood challenge with BTV, based on clinical signs and mortality, suggesting that the two viruses do cross-protect. However, Thomas[14] duplicated this experiment and reported the lack of cross-protection although the incubation period of the deer challenged with virulent virus was prolonged in comparison to that in the control deer.

Additionally, recent studies with BTV in sheep, goats and cattle[9] and with EHDV in elk[3] have shown that "immune" animals may still have virus

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circulating in the blood, and that this virus is closely associated with erythrocytes. These virus-erythrocyte viremias persisted, in most cases, up to 1 month after infection, at which time the studies were terminated.

To help clarify the status of these viruses in white-tailed deer, this study was undertaken to determine 1) the distribution of BTV and EHDV in the blood components of white-tailed deer, 2) if either virus persisted in the blood of the deer following the development of virus-specific neutralizing antibodies and if so, its effect on subsequent inoculation with homologous or heterologous virus, and 3) whether deer which are immunized to BTV or EHDV can withstand challenge with the virulent strain of the heterologous virus.

MATERIALS AND METHODS

Four adult white-tailed deer were used in this experiment. They were kept in tight isolation facilities at the University of Wisconsin Charmany Research Center. D-3 and D-5 were housed together as were D-13 and D-172. At the initiation of the study none of the deer possessed serum neutralizing antibodies to either BTV or EHDV.

D-5 was vaccinated with tissue culture adapted BTV strain BT-8 via three injections on days post-inoculation (DPI) O, 30 and 60. No virus was inoculated into D-3 as this animal was to serve as a contact control. On DPI 75, both D-3 and D-5 were challenged with 1.0 ml of $10^{4.0}$ tissue culture infective doses ($TCID_{50}$) of EHDV in infective deer blood.

The same experimental design was used with D-13 and D-172 except that D-172 was immunized with mouse-passaged EHDV strain EHDV-ND.[6] On DPI 75, both D-13 and D-172 were challenged with 1.0 ml of $10^{4.0}$ $TCID_{50}$ of BTV in infective deer blood.

All deer were observed and bled daily for 1 days following each inoculation. In the cases of the first and second immunizations, additional bloods were collected every other day until the 30th day post-inoculation. Blood samples were utilized for two purposes: first, to obtain serum samples, and second, for virus isolation attempts.

The serum samples were heat-inactivated, diluted to 1:40, and assayed for the presence of neutralizing antibodies to both EHDV and BTV using the virus plaque reduction techniques described by Thomas and coworkers.[15,17] The viruses were used at 30-80 plaque forming units and only those sera which caused a reduction of 50% or greater in plaque count were considered positive.

Blood samples for virus isolation attempts were mixed 1:1 with an anticoagulant solution of oxalate-phenol-glycerine (OPG)[3] which also served as a virus preservative. The blood samples were then fractionated by centrifugation into the various blood components, i.e., plasma-OPG, white-blood cells (WBC) and red blood cells (RBC).[8] All viral assays were done on monolayer cultures of BHK-21 cells.[8] Prior to inoculation of the cell cultures, the blood fractions were treated in a Branson sonifer with a ⅛ micro-horn for 30 seconds at an instrument setting of 4 and then diluted 1:10 in Hank's balanced salt solution.[8] The inoculated cultures were observed 7 days for development of cytopathic effect.

All viruses recovered from the deer were identified by neutralization tests with BTV and EHDV specific antisera.

RESULTS

Vaccination

The initial immunization injection in D-5 and D-172 with BTV and EHDV respectively, resulted in subclinical disease, viremia, and antibody response. BTV was recovered from the whole blood-OPG of D-5 from DPI 6 to 15 and in the blood components from DPI 5 to 22 (Table 1). The viremia was

[3] OPG-Potassium oxalate, 5 gm; phenol, 5 gm, glycerol, 500 ml; and double distilled water, 500 ml.

primarily cell associated, being first detected in the RBC component on DPI 5. Although the titers of virus associated with RBC fractions were consistently higher than those observed with WBC fractions, BTV persisted in both fractions for approximately the same length of time. All detectable virus disappeared within 8 days following the development of BTV neutralizing antibodies, which reached a peak titer of 1:1280 on DPI 24.

A similar situation occurred with EHDV in D-172. Virus was detected in the whole blood-OPG from DPI 7 to 10 and in the blood fractions from DPI 7 to 16 (Table 1). Again, the viremia was primarily cell associated with higher titers recorded for the RBC components. All detectable viremia disappeared within 6 days following the development of measurable EHDV neutralizing antibodies, which reached a peak titer of 1:640 on DPI 20.

No detectable viremia was recorded in D-5 or D-172 following the second and third immunizations of the homologous virus. By DPI 75, D-5 had a BTV serum neutralizing antibody titer of 1:2560 while D-172 had a titer of 1:640 to EHDV. Neither deer developed neutralizing antibody to the heterologous virus.

During this period, neither BTV nor EHDV viremia or antibody were detected in the contact control deer, D-3 and D-13.

Challenge

Following challenge with the virulent EHDV, D-3 died on DPI 9 (Table 2). The animal was viremic for 3 days prior to death; the highest virus titers being associated with the RBC fraction.

D-5 had a low level EHDV viremia from DPI 5 to 11. A detectable serum neutralizing antibody titer to EHDV was

TABLE 1. Distribution of BTV and EDHV in blood components of white-tailed deer; vaccine strains of viruses.

	D-5 (BTV)				D-172 (EHDV)			
DPI	Whole blood in OPG	Plasma	WBC	RBC	Whole blood in OPG	Plasma	WBC	RBC
0	—	—	—	—	—	—	—	—
2	—	—	—	—	—	—	—	—
4	—	—	—	—	—	—	—	—
6	3.2①	1.7	3.0	4.5	—	—	—	—
8	5.5	2.0	5.0	7.3	3.5	1.7	4.0	5.0
10	6.3	4.3	7.8	9.0	4.0	2.0	4.4	5.4②
12	4.0	3.8	5.8	7.3	—	—	4.3	5.3
14	1.5	—	4.0	6.0	—	—	2.5	3.8
16	—	—	3.8	6.3②	—	—	—	1.8
18	—	—	4.0	6.3	—	—	—	—
20	—	—	2.5	5.7	—	—	—	—
22	—	—	—	3.5	—	—	—	—
24	—	—	—	—	—	—	—	—
26	—	—	—	—	—	—	—	—
28	—	—	—	—	—	—	—	—
30	—	—	—	—	—	—	—	—

① Titers expressed at Log_{10} $TCID_{50}$ per 0.1 ml of inoculum.
② Virus-specific antibody first detected.

TABLE 2. Results of challenge test in white-tailed deer.

	Vaccination					Challenge					
		Viremia		Antibody Titer ①			Viremia		Death	Antibody Titer ②	
Deer No.	Virus	DPI ③	Titer ④	EHDV	BTV	Virus	DPI	Titer	DPI	EHDV	BTV
3	None	—	—	<10	<10	EHDV	6-8	7.0	9	<10	<10
5	BTV	5-22	9.0	<10	2560	EHDV	5-11	3.3	20 ⑤	640	2560
13	None	—	—	<10	<10	BTV	2-5	6.3	5	<10	<10
172	EHDV	7-10	5.4	640	<10	BTV	3-7	6.0	8	640	<10

① Antibody titer at DPI 75

② Antibody titer at death

③ Virus recovered from any blood fraction

④ Reciprocal of highest titer in RBC fraction

⑤ Euthanized, animal very sick

observed on DPI 9; there was no corresponding rise in the BTV antibody titer (Table 2). On DPI 18, D-5 was anorectic and depressed having a rise in rectal temperature of almost 2C. The deer was euthanized on DPI 20. Just prior to death, D-5 had serum neutralizing antibody titers of 1:2560 to BTV and 1:640 to EHDV. At necropsy, lesions of a hemorrhagic disease were observed and only EHDV was recovered from the spleen and liver of D-5.

Challenge of D-13 and D-172 with virulent BTV resulted in the death of the deer on DPI 5 and 8 respectively (Table 2). Both deer were viremic prior to death and again the highest virus titers were associated with the RBC fractions. Neither animal developed BTV-specific neutralizing antibodies, nor did D-172 have an amnestic response to EHDV.

DISCUSSION

The viremia patterns observed during this study in white-tailed deer are similar to those reported in other species infected with BTV or EHDV.[3,9] The virulent and non-virulent strains of each virus resulted in viremias which were primarily cell associated; the highest virus titers were detected in the erythrocyte component. Viremia in deer, however, differs from that in sheep, goats, cattle and elk[3,9] in that the deer lost detectable circulating virus within 2 to 8 days following the development of virus specific neutralizing antibodies. These antibodies subsequently did not confer protection against challenge with virulent heterologous virus.

Viremia can only be maintained if there is a continued release of virus into the blood or if there is an impairment of the virus clearance system of the body. Therefore, the length of time a cell associated viremia can persist is dependent on 1) whether the virus can replicate in the hematopoietic system and attach to newly formed RBC's and WBC's, and 2) whether the production of serum neutralizing antibodies is stimulated by the virus and whether these antibodies have access to the virus. Both EHDV and BTV can be isolated from the hematopoietic system of white-tailed deer with acute disease;[20] however, neither virus could be recovered from any tissue collected from deer possessing virus specific neutralizing antibodies.[5]

The coexistence of BTV and EHDV with their respective specific antibodies has been reported in sheep, goats, cattle and elk.[3,9,12] To explain this observation in sheep, goats and cattle, Luedke[9] hypothesized that BTV may seal itself within the plasma membrane of the erythrocytes similar to Sendai virus. Results in the present study, however, suggest that either 1) in deer blood, BTV and EHDV may be situated extracellularly and readily accessible to the neutralizing effect of the specific serum antibodies, or 2) that the viruses damage the blood cells of the deer causing their clearance from the circulation, while the antibodies prevent more virus from becoming attached to new blood cells.[19]

The failure of specific neutralizing antibodies to BTV or EHDV to protect the deer from cross-challenge with the heterologous virus may be the result of different antigenic strains of BTV. Neitz,[13] using a series of cross-challenge studies in sheep, recognized a plurality of antigenically different strains of BTV. Each virus strain produced a solid and durable immunity against itself, but only a variable degree of protection to challenge by heterologous strains. By means of virus plaque reduction cross-neutralization tests, Howell[6] classified 244 naturally occurring strains of BTV into 16 immunologically stable, distinct groups. Of these 16 groups, the type 10 group is known to occur in North America as established with BTV strain BT-8.[18] This is supported by the demonstration of a common immunological identity among various North American BTV isolates and BT-8.[16] However, the isolation of a BTV strain from Texas which is not neutralized by type 10 BTV antiserum has also been reported.[8]

To the best of our knowledge, based on cross-neutralization and cross-infection tests, there is only one antigenic type of EHDV, the New Jersey or NJ-55.[21]

Since EHDV does not cross-react with North American isolates (type 10) of BTV in various serological tests, it has been suggested that the two viruses are antigenically unrelated or only slightly related.[2,9,16,17] Recently, an EHDV-like virus has been isolated in Nigeria which cross-reacts in the complement-fixation test with both EHDV-NJ-55 and a type 6 BTV, suggesting that EHDV may yet prove to be a strain of BTV.[1,11]

When Vosdingh et al.[20] challenged two EHDV immune white-tailed deer with BT-8, both deer developed a viremia but survived the infection, whereas non-immune deer died. Thomas[14] repeated this experiment and his EHDV immune deer succumbed to the BT-8 challenge although death was delayed from 3 to 10 days. Both deer which Thomas challenged developed BTV-specific antibodies and one of these deer also had a gradual rise in its EHDV antibody titer. The results of the present investigation generally agree with those of Thomas.

Based on these results it would appear that the North American isolates of BTV and EHDV differ, at least partially, in their antigenic composition. However, it must be recognized that EHDV was being compared only to type 10 BTV and that comparisons to other BTV types, such as the type 6 may yield different results.

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