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Hematological and Virological Studies of Epizootic Hemorrhagic Disease of Deer*

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Abstract

The pathogenesis of epizootic hemorrhagic disease of deer (EHD) was studied in suckling white mice and white-tailed deer *(Odocoileus virginianus)* using these approaches: (1) measurement of hematological values, (2) assay of virus in various organs, and (3) detection of viral antigen within tissues using the direct fluorescent antibody technique. An increase in bleeding time was the only hematological change observed in mice. The virus content of the brains of infected mice increased rapidly during the early days of infection but little virus was found in other organs. Fluorescing viral antigen was detected only in the brains of acutely ill mice.

Infected deer had an increase in total erythrocyte counts as well as corresponding changes in erythrocyte-associated values. Although the percentage of neutrophils in the blood increased during infection, total leukocyte counts remained unchanged. Virus was isolated during the clinical illness from 13 of 16 organs tested. No viral antigen was demonstrated in any tissue by the direct fluorescent antibody technique.

Introduction

Since EHD was first described by Shope et al.[9] there have been several studies describing pathological changes and viral content of tissues of fatally infected deer[3,6,10]. This paper reports the results of studies on hematological changes as well as the location of EHD virus during the course of infection in both the natural host, the white-tailed deer, and a laboratory host, the suckling mouse.

The investigation was conducted in three parts: (1) the measurement of hematological values to determine if mouse hemostatic mechanisms, like those of the deer, are affected by the disease and if the disease could be tentatively diagnosed in either species by blood studies, (2) the assay of

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virus in various organs during the course of infection and (3) an attempt to detect viral antigen within the tissues by the direct fluorescent antibody technique to determine more accurately sites of virus replication.

Materials and Methods

Experimental animals and virus: Three-day-old white Swiss mice were inoculated intracerebrally with 70 mouse LD_{50} of mouse-adapted NJ-55 EHD virus originally isolated from deer by the late Richard E. Shope of the Rockefeller University. At 12 or 24 hour intervals mice were killed with chloroform, using the method of random numbers to dictate the order of harvest. At each interval two were taken for fluorescent antibody study; four additional mice were killed and organ pools of brains, hearts, spleens, kidneys and portions of lungs and livers were collected and stored at —65C until assayed for virus in suckling mice. The LD_{50}'s were determined by the method of Reed and Muench[7].

Bottle-raised male deer, approximately 3 months old, were inoculated intramuscularly with Alb-62-infected deer spleen suspension received from Lars Karstad of the Ontario Veterinary College, Guelph, Ontario. They were numbered and a random selection of numbers determined the order in which the animals were to be killed. This was done by the intravenous injection of 15 to 25 ml of saturated aqueous magnesium sulfate. Tissues were collected (Table 6) and stored at —65C until assayed in HeLa cells.

Tissue culture: Stock HeLa cell cultures were grown in M199 with 10% bovine serum. For viral assay, cells were inoculated in suspension by adding ½ ml of a 10^{-3} dilution of each deer tissue to HeLa cells in 60 x 15 mm plastic petri dishes (Falcon Plastics, Los Angeles, California) containing two 22 x 22 mm glass coverslips. The maintenance medium was Eagle's basal medium in Hank's balanced salt solution with 3% fetal calf serum added. It was changed on the day following inoculation and every 3 to 4 days thereafter. Eight days after inoculation a coverslip was removed, stained and observed for the presence of fluorescing cells — occurring either singly or in groups (foci). If none was seen, a second coverslip was taken 2 days later and similarly examined. To prepare coverslips for fluorescent staining, they were rinsed in PBS, allowed to air dry, fixed in acetone for 10 minutes at room temperature, and again allowed to air dry.

Hematology: (1) Mice. Suckling mice were tested daily for bleeding time, clotting time, total erythrocyte and leukocyte counts, and differential leukocyte counts. One EHD-infected and one uninfected litter (8 mice per litter) were used for each test.

The bleeding time was determined by slicing the tail and noting the time until blood could no longer be absorbed in a filter paper held close to the wound. The clotting time was established by filling a 0.6 mm capillary tube with blood and breaking it at intervals until a clot was detected between the broken ends. Total erythrocyte and leukocyte counts were performed in the standard manner[1] using Unopette disposable blood diluting pipettes (Becton, Dickinson and Co., Rutherford, New Jersey). One half the prescribed volume of blood was used when testing mouse blood. Blood smears for differential leukocyte counts were stained with Wright's stain and counted in the standard manner[1].

(2) Deer. Fawns were bled daily from a jugular vein from 2 days before inoculation until death. Disodium EDTA (ethylene-diaminetetra-acetate) was used as anticoagulant. Six hematological tests were performed to determine packed cell volumes, sedimentation rates, hemoglobin contents, total erythrocyte and total and differential leukocyte counts. All tests were performed using standard procedures[1]. Sedimentation rates were read at 30, 60, and 120 minutes and at 24 hours. Hemoglobin was measured by the cyanmethemoglobin method.

Fluorescent microscopy: Deer tissues were collected, sectioned and fixed in acetone by the method of Liu[4]. The fixed sections were stored at —20C in a dry atmosphere until stained.

The conjugate was made from the serum of a deer which had survived an experimental EHD infection and was subsequently hyperimmunized by a series of EHD virus inoculations over a 3 month period. The serum neutralized over 10^4 mouse LD_{50} of both NJ-55 and Alb-62 EHD virus in virus neutralization tests. The gamma globulin portion was obtained by the ammonium sulfate fractionation method of Coons[2]. Conjugation with fluorocein isothiocyanate was carried out by the modification of Marshall et al.[5] except that only 0.02 mg dye per mg protein was used. Excess fluorocein was removed by passage through a Sephadex G-50 fine column (Pharmacia, Uppsala, Sweden). After preparation the conjugate was adsorbed with either (1) mouse brain and liver powder before use on mouse tissue sections or (2) deer liver-spleen powder and bovine spleen-marrow powder before use on deer tissue sections[2]. A portion was also adsorbed with washed HeLa cells at 37C for 2 hours. Bright specific fluorescence could be readily demonstrated in HeLa monolayers infected with either virus used.

Tissue culture monolayer coverslips were stained with diluted (1:10) conjugate at 37C for 20 minutes. Following washing in PBS, they were mounted in buffered glycerol. Tissue sections were stained in the same way except that a 1:5 dilution of conjugate was used.

Blocking experiments were carried out as described by Liu[4] except that two applications of inhibiting antiserum were used rather than one.

A Zeiss fluorescence microscope equipped with a OSRAM HBO 220 V light source was used for examination of fluorescent preparations.

Results

Mice: Signs of illness were observed by the 3rd or 4th day after inoculation. All that were not killed during the experiment for tissue collection were dead by the 6th or 7th day after exposure. In addition to clinical signs of neuroptropism[11], necropsy revealed reduced amounts of milk in the stomachs of all mice from the time they first had overt signs of illness as compared to normal mice. In the later stages of illness large amounts of gas were observed in the stomach and intestines and a reduction in the size of the spleen was noted in approximately one half of the severely ill mice.

Of the 5 hematological tests used, only in the measurement of bleeding times did infected animals differ from the controls (Table 1). The mean bleeding time of both groups remained the same until 4 days after inoculation, at which time a 3-fold increase occurred in the inoculated animals. The decrease in the total leukocyte count of infected mice could not be considered significant because of the variation in the counts of individual mice within both groups at the time.

EHD virus was not detected in the majority of the mouse organs assayed (Table 2). However, within the 1st day after inoculation there was a 10^4-fold increase in virus titer in the brain. By the 2nd day maximum virus titer had been reached and remained at this level. Smaller amounts of virus were found in the lung pools and on the 5th day virus was found in all 6 organ pools. Assays were repeated on organ pools which were

found to contain virus. In all cases, the assays differed by no more than 0.5 LD_{50}.

No specific EHD antigen could be demonstrated by the direct fluorescent antibody method in sections of infected mouse lung, heart, liver, spleen or kidney. In brain sections only non-specifically fluorescing granulocytes were seen arranged along the path of the inoculating needle. Their numbers increased during the course of infection.

In a preliminary experiment an infected brain was sectioned so as not to include the inoculation trauma. Scattered cells were seen in the gray matter containing intracytoplasmic granules which fluoresced specifically with moderate intensity. Ten to twenty such cells (which appeared to be glial cells) could be seen in each section. The fluorescence could be blocked by unconjugated EHD antiserum and could not be observed when stained with conjugated normal deer gamma globulin.

Deer: Gross observation of EHD-infected deer at necropsy (Table 3) were similar to those reported by Shope et al.[10] and Karstad et al.[3].

A summary of the results of the hematological tests performed on deer is presented in Tables 4 and 5. Most of the values associated with erythrocytes remained normal for the first 4 days after inoculation. On the 5th and subsequent days, however, there was an increase in the total erythrocyte counts along with corresponding increases in amount of hemoglobin and packed cell volumes and a decrease in sedimentation rates (Table 4). Mean corpuscular volume and mean corpuscular hemoglobin values remained unchanged.

Although no change was noted in the total leukocyte counts during the course of infection (Table 5), the proportions of the major cell types did shift. The ratio of segmented neutrophils to lymphocytes remained fairly constant in all deer until the 5th or 6th days when the proportion of neutrophils increased with a corresponding decrease in lymphocytes. The proportions of monocytes, eosinophils and basophils remained relatively constant during the course of the experiment.

TABLE 2. *Assay for infective virus in tissues of suckling mice inoculated with EHD.*

	Day after inoculation							
Tissue	0	1	2	3	4	4.5	5	5.5
Brain	0[1]	5.6[2]	7.5	7.6	7.8	7.4	7.3	7.8
Lung	0	0	3.7	3.6	0	0	5.5	4.5
Heart	0	0	0	0	0	0	4.5	0
Liver	0	0	0	0	0	0	4.5	0
Spleen	0	0	0	0	0	0	4.0	0
Kidney	0	0	0	0	0	0	3.3	0

[1]0 = less than $10^{3.0}$ LD_{50}/gram.

[2]Log_{10} of infective virus/gram of tissue.

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TABLE 1. *A summary of hematological values of control and EHD-infected suckling mice*

Day after inoculation	Bleeding Time (seconds)		Clotting Time (seconds)		Erythrocytes (x 10^6/mm^3)		Leukocytes (x 10^3/mm^3)	
	Control[1] Litter	Test[2] Litter	Control Litter	Test Litter	Control Litter	Test Litter	Control Litter	Test Litter
1	81	90 (8)	60	60 (8)	3.1	3.2 (8)	4.6	5.0 (8)
2	73	56 (8)	60	54 (8)	2.9	3.7 (8)	3.7	4.0 (8)
3	71	77 (8)	60	56 (8)	2.9	3.4 (8)	4.4	4.1 (8)
4	71	221 (8)	60	60 (8)	3.7	3.6 (8)	3.5	3.2 (8)
5	78	265 (4)	56	63 (6)	3.6	4.0 (8)	4.2	2.4 (7)
6	NT[3]	AMD[3]	58	75 (3)	3.8	4.1 (4)	4.3	2.4 (4)
7	NT		NT	AMD	NT	AMD	NT	AMD

[1]Average of eight mice in each control litter
[2]Average of number of mice in parentheses
[3]NT = Not tested; AMD = All mice dead.

(continued from page 80)

TABLE 3. *A summary of gross observations of EHD-infected deer at necropsy*

Deer No.	Day after Inoculation	Overt Signs	Reddening of Oral Mucosa	Surface hemorrhages				Congested Kidney	Excess Pericardial Fluid
				Liver	Spleen	Heart	Intestine		
99	0	—[1]	—	—	—	+	—	—	—
103	1	—	—	—	—	—	—	—	—
546	2	—	—	—	—	—	—	—	+
117	3	—	—	—	—	—	+	—	+
566	4	—	—	—	—	—	—	—	—
45[2]	5	+	—	++	++	++	++	++	++
507[3]	6	++	++	++	++	++	++	++	++
23[4]	7	++	++	—	++	—	++	—	+
174[5]	6	—	—	+	—	+	—	+	+

[1] — = No signs or lesions; + = Mild signs or lesions; ++ = Severe signs or lesions.
[2] Additional hematuria
[3] Additional hemorrhages on rumen and abomasum
[4] Additional hemorrhages on rumen and bladder
[5] Found dead on day 6. Appeared normal the previous day.

TABLE 4. *A summary of erythrocyte-associated hematological values of EHD-infected deer.*

Day After Inoculation	Number Tested	Sedimentation Rate (mm/24 hours)	Packed Cell Hemoglobin (gm/100 ml)	Packed Cell Volume (Percent)	Total Erythrocytes (x 10^6/mm^3)	Mean Corpuscular Volume (u^3)	Mean Corpuscular Hemoglobin (uugm)
—1	5	4.7[1]	17.0	48	14.7	33.3	11.6
0	8	4.9	15.8	48	15.0	32.0	10.6
1	7	5.2	15.3	45	14.5	31.0	10.5
2	7	4.9	15.0	45	15.3	29.4	9.6
3	6	4.7	16.5	48	15.3	31.4	10.8
4	5	3.9	17.2	49	16.3	30.0	10.4
5	4	2.0	20.6	60	19.2	31.2	10.7
6	2	2.2	22.4	67	20.5	32.6	10.9
7	1	0.9	24.3	66	21.0	31.4	11.6

[1] Data recorded as mean values of the deer tested.

TABLE 5. *A summary of leukocyte values of EHD-infected deer*

Day after Inoculation	Number Tested	Total Leukocyte (x 10^3/mm^3)	Differential Leukocyte (percent) Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils
—1	5	2.5[1]	68	23	6	1	0
0	8	3.6	65	25	8	1	1
1	7	3.6	63	30	7	1	1
2	7	2.8	62	30	5	1	1
3	6	2.8	53	35	9	1	1
4	5	1.9	68	24	5	1	1
5	4	3.0	87	10	3	1	0
6	2	4.3	84	13	4	0	0
7	1	1.9	NC[2]	NC	NC	NC	NC

[1] Data recorded as mean values of the deer tested
[2] NC = Not counted

TABLE 6. *The presence of infective EHD virus in deer tissues as measured by the fluorescent focus method.*

Tissue	Day after inoculation 0	1	2	3	4	5	6	7
Spleen	—[1]	—	—	—	—	++	++	++
Liver	—	—	—	—	—	+	++	+
Heart	—	—	—	—	—	NT	+	+
Lung	—	—	—	—	—	—	+++	++
Kidney	—	—	—	—	—	+	++	++
Pancreas	—	—	—	+	—	+++	+++	+++
Adrenal gland	—	—	—	—	—	++	++	++
Bladder	—	—	—	—	NT	+	+	+
RFLN[2]	—	—	—	—	—	+	++	++
LFLN[2]	—	—	—	—	—	—	++	++
Bone Marrow	—	—	—	—	—	+++	+++	+++
Testis	—	—	—	—	—	+	+	+
Whole Blood	—	—	—	—	—	+	+	—
Muscle	—	—	—	—	—	—	—	—
Brain	—	—	—	—	—	—	—	—
Spinal Cord	—	—	—	—	—	—	—	—
Feces	—	—	—	—	—	—	—	—

[1] — = < 1.2 x 10^4 focus forming units/gram of tissue
+ = 1.2 to 6.0 x 10^4 focus forming units/gram of tissue
++ = 6.1 to 12.0 x 10^4 focus forming units/gram of tissue
+++ = > 12.0 x 10^4 focus forming units/gram of tissue
NT = Not tested

[2] RFLN = Right femoral lymph node; LFLN = Left femoral lymph node.

The presence of EHD virus in deer tissues as measured by the tissue culture fluorescent focus method is summarized in Table 6. No virus was found in any tissue until 5 days after inoculation with the single exception that the pancreas of the deer killed on the 3rd day contained EHD virus.

Of the positive tissues, those which had the most virus were the pancreas, bone marrow and lung (more thas 12 x 10^4 focus forming units (FFU) per gram of tissue). Intermediate amounts of virus (6.1 to 12 x 10^4 FFU per gram) were detected in the spleen, liver, kidney, adrenal glands and lymph nodes; small amounts of virus (1.2 to 6.0 x 10^4 FFU per gram) were found in whole blood, heart, bladder and testes. Virus was not detected in the brain, spinal cord, muscle or feces.

Other than these general groupings, no attempt was made to quantitate the amount of virus in each tissue. Since fluorescent focus counts of more than 20 per coverslip were rare and the majority were below 10, detailed quantitation was not considered appropriate.

No EHD antigen was demonstrated in any tissue section stained by the direct fluorescent antibody technique. Scattered non-specifically fluorescing granulocytes were observed in sections of the spleens, livers, and

lungs of animals killed 4 to 7 days after inoculation but not in animals killed earlier in the infection. These cells fluoresced equally well with both conjugated normal and immune sera. They were identified by staining the sections with hematoxylin and eosin stains. In the lung particularly these cells were seen surrounding small blood vessels. Granulocytes were seen in lymph nodes earlier in the infection. Many were seen in sections of the right femoral lymph node during the first 3 days of infections and in the left femoral lymph node on the 2nd and 3rd days.

Discussion

While there is evidence that the white-tailed deer in this study developed a generalized infection, the inoculation of the laboratory host, the suckling mouse, by the intracerebral route, appears to result in a more limited infection. Hematological values normally associated with generalized disease such as changes in total and differential leukocyte counts were not found in mice during the course of the fatal infection. Assay of infectious virus in organ pools also suggests the occurrence of a limited infection in suckling mice.

The lack of demonstrable change in clotting times of infected mice also suggests that EHD infection in mice differs from that in deer in which hemostatic defects are pronounced.

EHD virus replicates rapidly in the brains of intracerebrally-infected suckling mice. Within 1 day after inoculation a 10'-fold increase in virus had occurred. Maximum virus titers in the brain were reached from 1 to 2 days before the mice had overt signs of disease.

It appears that virus was present in low concentrations in the lungs of infected mice. The titers demonstrated early in infection were just within the sensitivity of the test. This sensitivity was limited by the amount of inoculum which could be administered to a mouse.

In light of the hematological evidence which points to a limited infection, the presence of virus in all the mouse organs assayed 5 days after infection suggests a release of virus from the area(s) of replication and its dissemination throughout the host, probably through the vascular system. If this release did occur, it is not clear whether it was an exceptional instance or the general case. Since the organs of four mice were pooled for each assay, this release of virus need have occurred in only a single animal in order to be found in all the organ pools. Aseptic necropsy procedures reduced the possibility of any transfer of virus between organ pools.

Evidence that the white-tailed deer in this study sustained a generalized systemic infection is illustrated by the occurrence of virus in most organs asd by the pronounced disease condition reflected in the hematological values obtained during the course of infection.

Most pronounced in the deer hematological data was the increased erythrocyte counts. These occurred earlier and rose to a higher level than

had been reported in previous studies[3]. Parallel to the increased erythrocyte counts were increases in packed cell volumes. The constant values of the mean corpuscular volumes provided evidence that these increases were not because the erythrocytes became larger but only that greater numbers were present. It also rules out any increase of reticulocytes, a much larger, young erythrocyte often associated with hemolytic diseases.

The increase in hemoglobin was also a reflection of the increase in numbers of erythrocytes, as the mean corpuscular hemoglobin values remained unchanged during the infection, indicating no increase in the amount of hemoglobin per erythrocyte.

It is not known if the increase in erythrocytes is because of an accelerated production of these cells or because of hemoconcentration. No increase in reticulocytes was detected and no immature nucleated erythrocytes were observed in blood smears, indicating that an abnormally high rate of cell manufacture was probably not occurring. As a result of these findings and in light of the pathological changes which take place in the vascular system of EHD-infected deer, the notion of hemoconcentration is more plausible.

As in this study, Karstad et al.[3] found no changes in the total leukocyte counts during the course of the disease in deer. However, they did report a high percentage of lymphocytes in the blood of uninfected deer in contrast to the higher percentage of neutrophils detected in this study. In both studies an increase in neutrophils and a corresponding decrease in lymphocytes were noted in the terminal stages of infection. Neutrophilia is associated with a variety of conditions including hemolytic crisis and significant blood loss[8]. Deer in the terminal stages of EHD appear to experience significant loss of blood from the vascular system into the surrounding tissue which may explain the leukocyte shift.

The findings of this study did not result in any new method for the early detection of EHD infection by hematological means in either host. The day on which changes in the blood picture were first detected was usually the same day on which animals began to show signs of disease.

A more sensitive method is required for the assay of EHD virus in deer tissue. Even when inoculated with tissues which have been reported to contain high levels of virus[6], very few fluorescing foci were detected in HeLa cell monolayers. While almost every tissue tested, except those of the nervous system, were found to contain infective virus in the later stages of disease, there was no great differences in the amount of virus per gram of tissue. Titers were far less than the 10^7 $TCID_{50}$ reported by Mettler et al.[6] in HeLa cell tube titrations of EHD-infected deer tissues.

Further evidence of the insensitivity of the assay system is that only a single tissue — the pancreas — was shown to contain infective virus prior to the onset of clinical signs of EHD. It would appear that viral replication and dissemination must take place somewhere during the first 4 days of infection but the site could not be determined with the assay system used.

The inability to demonstrate specific fluoresence in EHD-infected mouse and deer tissues was disappointing. It is difficult to explain the lack of consistent staining of viral antigen, particularly in mouse brain which contained more than 10^7 mouse LD_{50} of virus. The conjugate had been shown to be potent for the staining of infected tissue culture monolayers. The same fixation and staining procedures were used in preparing tissue sections except that a more concentrated conjugate was used. One would expect that it would not be difficult to demonstrate viral antigen in mouse brain which contains such large amounts of virus. It may be that the proper deer tissues were not examined. Although many of the tissues studied were known to contain infective virus, the situation may be similar to that found by Stair (personal communication) in bluetongue infection. Visceral organs containing infective virus show little fluorescence while such tissues as nasal mucosa, tongue and the gasserion ganglia had prominent fluorescing cells.

A more complete study of the pathogenesis of EHD must await a more sensitive method for detecting and quantitating EHD virus within host animal tissues.

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