ELECTRON MICROSCOPE STUDIES AND PHYSICOCHEMICAL CHARACTERISTICS OF EPIZOOTIC HEMORRHAGIC DISEASE VIRUS

by

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INTRODUCTION

Epizootic hemorrhagic disease (EHD) was diagnosed in white-tailed deer (Odocoileus virginianus) in 1955. In this first reported case almost 700 deer were destroyed as a result of this disease. EHD probably occurred much sooner than this, since similar pathological signs and lesions indicate that the same disease had been known to occur in many instances dating back to 1890. To date, this disease has been reported in many different areas such as New Jersey, Michigan, North Dakota, South Dakota, and Alberta. The disease usually appears suddenly in a population of deer inhabiting a relatively small geographic area and then, just as quickly, completely disappears from the same population.

Bacteriologically sterile filtrates from deer tissue infected with EHD have been used for passage into suckling mice with subsequent isolation of virus from the brain. There has been some indication that this virus is a reo-like virus, that is, containing double stranded RNA with an icosahedral type of morphology. Seriologic studies of various strains of EHD virus indicate that there are some similarities between different strains of EHD. However, some reports have indicated that there are two serotypes, the New Jersey and the South Dakota type.

This study was undertaken to obtain EHD virus from suck-

ling mouse brain and adapt it to cell culture for high titer growth. Subsequently a purification method was to be determined to obtain virus for electron micrograph studies and the determination of physicochemical properties.

LITERATURE REVIEW

The New Jersey and the South Dakota strains of EHD were first serially transmitted in white-tailed deer by Shope et al. (45). Initially a 10% suspension of spleen, liver, and kidney were used inocula. Subsequently, it was shown that spleen provided the more infective inoculum. Similar transmission has been reported by Pirtle et al. (40). Both groups used as inocula a 10% bacteriologically sterile suspension of infected tissue that had been passed through Berkefeld filters. Attempts to infect other animals such as rabbits, guinea pigs, hamsters, swine, sheep, calves, and goats have failed.

Shope et al. (45) and Pirtle et al. (40) described the salient pathologic characteristics as petechia and ecchymoses in the axillary and inguinal areas and hemorrhage throughout the viscera of the animal. The peritoneal cavity usually contained an excess of clear straw-colored fluid. The spleen, liver, and kidneys were generally dark and congested; the probable cause being excessive hemorrhage. Extensive hemorrhages appeared to be due in part to inability of the blood to clot. Karstad et al. (21) reported that the clotting time changed from the normal 3 to 5 minutes to 23 minutes just before death of deer inoculated with the South Dakota strain of EHD. Similarly, prothrombin time had markedly increased.

Tsai et al. (48) did electron micrograph studies of the

pathogenesis of EHD virus, in which they demonstrated a focal development of moderately electron-dense materials or viral matrices. Also, they showed virus particles and tubules in the cytoplasms of endothelial cells of small blood vessels suggesting that EHD virus infects endothelial cells. They concluded that viral injury to endothelial cells may initiate a series of events, including intravascular coagulation, which may result in hemorrhages. Platelets obtained from blood of infected deer contained virus-like particles, but were otherwise unaltered. Similarly, Tsai et al. (48) reported that viral formation in BHK-21 cells and neurons of infected mouse brains was associated with the development of foci of moderately electron-dense materials. These foci or viral matrices consisted of granular elements and were observed in the cytoplasm near the nucleus. Also, tubular structures were sometimes found in these cells which closely resembled the tubular structures reported in endothelial cells of infected deer.

EHD virus has been reported by Shope et al. (45) to be stable for at least 9 months when stored frozen in CO_2. Infected tissues were stored in 5 to 10% suspensions in saline or 50% glycerol-saline. The virus was equally resistant to degradation in either solution. Also, EHD virus has been shown to be relatively sensitive to sodium deoxycholate treatment as described by Mettler et al. (34).

Propagation of different strains of EHD virus in other

animals and *in vitro* has been reported by several investigators. Initially, Mettler et al. (34) reported growth of New Jersey strain of EHD in suckling mice and HeLa cell culture. Mice injected intracerebrally with deer tissue suspension had "mild convulsions, tremors, and irregular respiration by the seventh day postinoculation". This occurred in about one-half of the infected population from the initial passage of deer tissue. In subsequent passages of mouse brain tissue the animals had the same signs by the fourth day. Depending on the dilutions of the inoculum, HeLa cells infected with the same deer tissue suspension had cytopathogenic changes between 4 to 8 days after inoculation. Routinely, Tsai et al. (48) used BHK-21 cells with considerable success for high titer growth. Recently, EHD virus isolated from infected animals has been propagated in Vero continuous cell culture by Bando (3). Cytopathogenic effects were observed in these cells within 10 to 13 days.

Based on filtration experiments and electron micrograph studies, Pirtle et al. (40) reported that the South Dakota strain of EHD virus was roughly spherical and about 20 to 30 nm in diameter. After extensive electron micrographic examinations Tsai et al. (48) reported that the Alberta strain of EHD virus was 60 nm in diameter and had cubic symmetry. The surface of full capsids were composed of capsomeres which contained an axial hole about 4.5 nm in diameter.

Some discrepancy exists in the serological comparison of EHD virus to bluetongue virus (BTV), and in the comparison of various

EHD virus strains with each other. Thomas et al. (47) demonstrated by plaque reduction test that BTV and EHD virus are completely dissimilar antigenically. Plaques resulting from EHD virus were smaller "(pinpoint to 1 mm), irregular shaped," taking 7 days to form. BTV plaques were larger, circular shaped, taking only 3 days to develop. Also, complement fixation and agar gel precipitin tests failed to exhibit any cross reaction between the two viruses. Morphologically the two viruses are similar in size, shape, and tubule formation in infected cells. However, Moore et al. (38) demonstrated antigenic similarities between BTV isolated from Culicoides mosquitoes and the New Jersey strain of EHD virus. Using complement fixation tests, several different strains of BTV were neutralized by immune mouse ascitic fluid for EHD virus. Some isolates were actually from pools of Culicoides and therefore, may or may not have contained mixtures of various types of BTV and/or EHD virus.

Cross-neutralization tests performed by Shope et al. (45) in deer have indicated serological differences between the South Dakota and the New Jersey strains of EHD. Using serum from deer which have survived infection with either strain of virus, it was found that the homologous strain of virus was neutralized, but the heterologous strain was still lethal. If a reduction in titer occurred it was not detectable because of an insufficient number of test animals.

Wilhelm et al. (56) reported that the Michigan, North Dakota,

New Jersey, and Alberta strains of EHD viruses were antigenically similar. Immue serum prepared in rabbits to each virus isolate neutralized the lethal effect of the homologous and each of the heterologous strains of the virus when mice were used as test animals.

Recently, Tsai et al. (49) demonstrated that the Alberta strain of EHD virus is a double-stranded RNA virus, with a melting point of 94 C in 0.1 x SSC. Replication of the virus was not inhibited by 5-bromo-2'-deoxyuridine or 5-fluoro-2'-deoxyuridine. The buoyant density in CsCl was 1.38 g/ml.

MATERIALS AND METHODS

Virus

The EHD virus obtained was from the sixth passage in mouse brain, EHD strain-10 (SD_{10})*. It was received at relatively low titer, approximately 10^{2}, and did not contain detectable bacteria or mycoplasma.

To increase titer the virus was passed initially in mice 12 to 36 hours old. Each mouse was injected intracerebrally with 0.05 ml of inoculum. Mice were observed for pathologic signs for 5 consecutive days, after which each mouse was frozen at -76 C. However, most had signs of irregular respiration, cyanosis, and convulsions after approximately 80 hours and at that time the mice were frozen.

Mouse brains were excised from the initial passage and placed in mouse brain solution (MBS) which consisted of 16 ml saline (0.86% NaCl), 0.1% bovine serum albumin, 1 ml antibiotics (10,000 units penicillin/ml, 1,000 units mycostatin/ml, 10 mg streptomycin/ml). A total of 20 ml of MBS per 1 ml of mouse brain was placed in a Waring blender and blended for 4 one minute intervals at 5 C. Between each blend the suspension was immersed in an ice bath for one minute. This procedure was repeated for

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*From G. C. Parikh, Associate Professor, Department of Bacteriology, South Dakota State University, Brookings, South Dakota.

5 successive mouse passages, with subsequent inoculation of mouse brain suspension from the fifth mouse passage into African green monkey kidney* (Vero) cells, passage 132. Growth media for both stationary and roller bottles of Vero cells consisted of Eagle's minimum essential medium with Hanks' balanced salt solution (HMEM), 8% calf serum, 1 X antibiotics (100 units penicillin/ml, 10 units mycostatin/ml, 100 ug streptomycin/ml), and 0.35% sodium bicarbonate. Cell cultures were used after confluent monolayers formed at approximately 18 hours. The medium was decanted and a 20 ml inoculum of mouse brain tissue was added to the cells for a period of 3 hours. The inoculum was removed and Eagle's minimum essential medium with Earle's balanced salt solution, 1.5% of gamma globulin (G-G) free calf serum, 1 X antibiotics, and 2.2% sodium bicarbonate was added. The medium was changed after 72 hours and the cells harvested between 7 and 9 days postinoculation. Time at which cells were harvested was determined by microscopic and macroscopic examination of cells and cell culture fluid. Cells that remained attached to glass surfaces were removed by freeze - thawing (usually 1 X) and the suspension was centrifuged at 4080 x g for 30 minutes. The pellet was resuspended in two volumes of phosphate buffered saline (PBS) and stored at -76 C. The supernatant was used for subsequent Vero cell inoculation in roller bottles.

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*From Norden Laboratories, Lincoln, Nebraska. American Type Culture Collection Cell Repository Designation CCL81.

When supernatant was used as inoculum, it was placed on cell cultures for 18 hours and the medium was not changed after 72 hours. Also, the inoculum was added just prior to confluent monolayer formation. Cells were harvested 5 days after inoculation.

Virus Assay

Virus titer of both the cell debris and supernatant was determined separately. Tubes of Vero cells were seeded at 275,000 cells/ml/tube, and a confluent monolayer formed after 18 hours. Media used for growth and maintenance of cells was the same as described for roller bottle growth of Vero cells. Tenfold dilutions of each sample were made ranging from 10^{0} to 10^{-7} and 5 tubes were inoculated with each dilution. Dilution fluid used to resuspend cellular debris consisted of 1 X PBS with 0.5% G-G free calf serum and 1 X antibiotics. Tubes were examined microscopically at 12 to 24 hour intervals for cytopathogenicity (CPE). Titers of virus were determined according to the Reed and Muench (41) method. Control tubes of Vero cells contained the same passage of cells and were incubated with dilution fluid during the viral inoculation time.

Cytopathogenicity

CPE was examined microscopically in tubes and roller bottles. Also, cells were harvested from roller bottles 5

days after inoculation of EHD virus, prefixed with gluteraldehyde, postfixed in osmium tetroxide, dehydrated in a graded series of alcohol concentrations, and embedded in an Epon 812 - Araldite 502 mixture (37). Thin sections prepared were examined by an RCA-EMU3 or a Siemens Elmiskop.

Host Range

Besides the initial growth of EHD in suckling mouse brain tissue the virus was grown *in vitro* using either Vero or BHK-21 cells. Other methods of growing EHD virus such as using deer kidney cells and embryonating hen's eggs inoculated by various routes has been attempted with no success (45).

Purification of EHD Virus

Purification procedure used was a modification of Verwoerd's (51). Suspensions of EHD virus grown in roller bottle cultures of Vero cells were centrifuged at 4080 x *g* and pellets resuspended in 1 X PBS buffer (5 ml buffer/2 ml cell debris) and 0.001 *M* chymotrypsin. An equal volume of trifluorotrichloroethane* (Genetron-113) was added to the above suspension and mixed for 5 minutes with a vortex mixer at 25 C. Subsequently, the suspension was sonicated for 30 seconds in a L & R ultrasonic 320 D

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*Allied Chemical. 1496B-62. General Chemical Division. New York, N.Y.