Isolation and Characterization of Virus From Horses Infected With Equine Infectious Anemia.

Virginia Ann lewis Boyd

Louisiana State University and Agricultural & Mechanical College

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The Louisiana State University and
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ISOLATION AND CHARACTERIZATION OF VIRUS FROM HORSES INFECTED WITH EQUINE INFECTIOUS ANEMIA

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in The Department of Microbiology

by

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ABSTRACT

Equine Infectious Anaemia (EIA) virus was isolated from infected serum and tissue culture fluid from bone marrow cells (BMC) by sucrose and CsCl equilibrium density-gradient centrifugation. The virions were further purified and characterized. The purified virus fraction had a density of 1.146 g/cm³, measured 70-120 nm in diameter with 45-50 nm nucleoid, and when inoculated into a normal horse produced an acute infection of EIA. The nucleic acid extracted from the purified virus preparation was a linear, double stranded DNA molecule.

BMC allowed viral propagation and electron microscopic (EM) observation of thin sections of infected cells revealed two morphologically distinct virus types. Type I virus had a 80-120 nm diameter with a well defined outer membrane. Type II virus measured 70 nm in diameter with an electron dense nucleoid 45-50 nm in diameter with an electron translucent core and electron dense outer coat. Both virus types were found in infected cells and infected serum. Similar particles were not detected in uninfected cells or normal horse serum.

Detection of the virus in BMC was most successful with IFA techniques. Fluorescence in the infected cells was primarily cytoplasmic. Occasionally early infected cells
were observed with fluorescence at the nuclear membrane and at the cell's surface in late infection. The percentage of cells which were harboring virus was low as also indicated by EM studies.

Antibodies to ETA were demonstrated by immunodiffusion. Intact virions and ether treated virions were reactive antigens in the immunodiffusion tests.

The following characteristics were observed for viral propagation by EM observation: margination of chromatin, cytoplasmic vesicles containing two viral types, budding of virus from cytoplasmic vesicle and cell surface, envelope and DNA content of virus, and low percentage of infected cells.
INTRODUCTION

This investigation was conducted to compare the virions found in serum and bone marrow cells from horses infected with EIA with virions previously reported to be involved in EIA. Characterization subsequent to isolation and purification of the virions was made by electron microscopy, nucleic acid and density determinations, and tissue culture studies on the replication of the viral isolates.

To date two viral isolates have been incriminated as the causative agent of EIA. The type I particle measured 80-120 nm in diameter with a 45 nm electron dense core. This particle was reported to have a density of 1.146 g/cm³ (Nakajima et al., 1969a) and contain RNA (Nakajima et al., 1970). Henson et al. (1969) reported this type I particle could be observed in normal and infected horse leukocyte cultures but only the type II particles could be found in cells infected with EIA. The type II particle measured 70 nm in diameter and contains an electron translucent core. Subsequent characterization of the type II particles were not reported when this investigation began. It was proposed to demonstrate these two virus types in bone marrow cells and to carry out further characterization of the isolated virions.

Immunological techniques provided a means for detection of the EIA virus in infectious serum and tissue culture
cells. Immuno-diffusion tests using purified virus was used as the antigen in testing the EIA antibody level of various antisera. Fluorescent antibody techniques and EM studies provided an efficient method for detecting the presence of virus in infected tissue culture cells. Ultimate identification of the infectivity of the isolate as an EIA agent was made by sub-inoculation into a normal horse with the subsequent production of an acute infection.
A. Equine Infections Anemia, General Pathogenesis

Equine infectious anemia (EIA), or swamp fever is an acute or chronic infectious disease of horses. It is characterized principally by intermittent fever, marked depression, icterus of the mucous membranes and anemia.

The course of the disease is generally progressive and is characterized by prolonged periods of clinical remission despite a persistent viremia. This is unlike the response to most mammalian viral infections; however, it resembles more closely the etiologic agents considered in the slow or latent virus category; i.e. sarcoid, lymphocytic choriomeningitis (LCM), Aleutian disease (AD), and Marek's disease (MD) (Abinanti, 1967; Henson et al., 1969). The response may represent either a type of immunologic tolerance to viral antigen, a prolonged immune response to virus-altered components, or a virus-induced mutation or transduction of immunologically competent cells (Squire, 1968).

Information concerning the pathogenesis of the lesions in EIA has accumulated slowly since the early descriptions of tissue alterations (Henson et al., 1969). The macroscopic lesions in infected horses vary depending upon the stage of the disease, but generally include anemia, accentuated
hepatic lobular architecture, enlarged splenic, hepatic, and renal lymph nodes and widespread hemorrhages (Dreguss and Lombard, 1954; Ishii, 1963; Henson et al., 1969; Ishitani, 1966). Microscopically, the pathology is characterized by generalized lymphoproliferative changes with perivascular and hepatic lymphoid infiltrations, lymphoid hyperplasia in the lymph nodes and spleen, hepatic cell necrosis, glomerulitis and hemosiderosis (Dreguss and Lombard, 1954; Henson et al., 1969; Ishii, 1963; Squire, 1968). During active disease mononuclear infiltration and necrosis in the liver are usually quite prominent.

Relatively little is known about the mechanisms responsible for these lesions. That there is little direct cytolytic effect, except in hepatic tissues, suggests the virus has a relatively small influence and indicates at least two mechanisms that may act together causing lesions in EIA. EIA-infected horses are almost continually viremic (Stein et al., 1955) with the amount of virus increasing during the febrile periods (Kono, 1969). It has also been shown that infected horses produce antibodies directed against the virus (Henson, Gorham, Kobayashi, and McGuire, 1969; Kobayashi et al., 1969; Kono and Kobayashi, 1966). One possible mechanism for the phlogistic reaction would be the consequence of a virus-antivirus antibody complex localizing in the tissues and fixing complement. The result would be induced tissue damage and cellular infiltration such as is evidenced in glomerulitis that occurs in AD of mink (Henson, Gorham,
Padgett, and Davis, 1969) and mice infected with LCM virus (Tanaka and Sakaki, 1962).

The second possible mechanism involves the direct cytolytic effect of lymphocytes when they come in contact with an antigen to which they are sensitized. Speel et al. (1968) have shown that when sensitized lymphocytes contact cells containing the antigen, the latter cells (target cells) are killed. If EIA virus were present in the tissues an influx of sensitized lymphocytes would be stimulated. The virus either within the target cells or only in close association with them would result in lymphoid cell infiltration and cell death (Granger and Kolb, 1968; Ruddle and Workman, 1968).

B. Immunological Characteristics of EIA

Many attempts have been made to diagnose and detect EIA. Efforts with immune adherence (Saurino et al., 1966), indirect passive hemagglutination (Saurino et al., 1966), hemagglutination (Dreguss and Lombard, 1955), precipitation (Moore et al., 1966; Russell et al., 1966), complement fixation (CF) (Kono et al., 1966) and gel diffusion (Henson et al., 1969) were not satisfactory or reproducible.

Kono and Kobayashi (1966) utilized antigen produced in EIA-infected horse leukocyte culture (HLC) in a CF test. The production of antigen in vitro was shown to be proportional to the level of infectivity of the inoculum (Kono, 1968). This antigen was shown to be intimately related to
the virus particle although it could be separated from the complete virus by ether treatment. Henson et al. (1969) have shown that the CF antigen may be separated from the infectious particle by ion exchange chromatography. This test has limited application since CF antibody does not remain elevated throughout the course of the disease (Henson et al., 1969). Antibody becomes detectable 17-21 days after inoculation and persists 62-70 days postinfection. The CF antibody then declines and becomes undetectable and is not recalled during subsequent exacerbations of clinical disease (Henson et al., 1969; Kono, 1968).

Tests have also demonstrated a lack of cross reactivity between the CF antigen in EIA with antibodies produced against equine rhinopneumonitis virus, cytopathic equine orphan virus, equine arteritis virus, and other equine herpes viruses (EHV) (Kobayashi and Kono, 1967b; Kono and Kobayashi, 1966). This is very important since a large proportion of the equine population is infected with EHV (Kobayashi and Kono, 1967b).

A precipitin test which detected circulating antigen in the serum of horses infected with EIA was developed by Moore et al. (1966) and Russell et al. (1966). Close correlation was observed between positive precipitin tests and clinically apparent EIA infections; however, it has been reported that this test results in both false positive and false negative reactions (Altara et al., 1953).
Saurino et al. (1966) identified serum from EIA-infected horses on the basis of results obtained with indirect passive hemagglutination and immune adherence tests. The efficiency and reliability of these tests were low, however.

El-Zein et al. (1968) reported that when an equine dermal cell line was infected with serum from an EIA-infected horse, plaque reduction was obtained in a vesicular stomatitis virus challenge infection. Whether or not this test is specific remains to be shown.

Henson et al. (1969), Henson, Gorham, Kobayashi, and McGuire (1969), Kobayashi et al. (1969), Kono et al. (1969) and Tokui et al., (1968), demonstrated neutralizing activity in the serum of some EIA-infected horses. Horse inoculation studies (Henson, Gorham, Kobayashi, and McGuire, 1969a; Kobayashi et al., 1969, Kono, 1969; Stein and Gates, 1950; Stein et al., 1955; Tokui et al., 1968; McGuire et al., 1969) suggested that CF antibodies occurred earlier and persisted for a shorter time period than did neutralizing antibodies. Neutralizing antibodies became detectable about 30 days after inoculation and persisted longer than 549 days; however, all horses did not respond. The role of this antibody in the disease and as an indicator system remains to be evaluated.

Once a tissue culture system of either bone marrow or horse leukocytes was established in which EIA virus was propagated techniques were available for EIA virus detection. One such technique was immunofluorescence (IF). Ushimi et al.
(1970) reported EIA viral specific antigen could be demonstrated in HLC by the indirect IF technique. Leukocytes were taken from suspension culture and inoculated with Wyoming strain of EIA virus at the fiftieth passage. Fluorescence of the infected cells was confined almost entirely to the cytoplasm. Occasional bright fluorescence of the cell surface was consistent with virus budding at the cell membrane as observed in electron microscope (EM) studies. The use of IF does not appear practical as a rapid diagnostic tool because cultures of horse leukocytes or bone marrow must be grown and a relatively long time is necessary for subsequent testing (Ushimi et al., 1970).

Fluorescent antibody studies could detect fluorescence along the membranes of the nucleus within the first 5 hrs and subsequently in the cytoplasm and along the cell membrane (Coggins and Norcross, 1970). This was also confirmed by Crawford (personal communication) who reported 15-50% cells to be infected.

Success has been obtained using immunodiffusion for the detection of EIA-positive serum. Coggins and Norcross (1970) recently reported that an antigen appears in the spleen of acutely-infected ponies which is detectable by the agar gel-immunodiffusion test. Antibody against this antigen, which seems to be specific for EIA appeared as early as 18 days after inoculation and remained in the serum of a few ponies for more than 3 yrs after infection. Normal animals tested had no detectable antigen or antibody specific for
EIA. Results of immunodiffusion tests by others are consistently accurate for detection of EIA-positive serum (Norcross; Roth; Hansen; personal communication). Attempts to concentrate and purify the antigen from the spleen have been successful as shown by an increase in the sharpness of the precipitate arcs obtained (Norcross; Roth; personal communication). This test is the most promising for detection of EIA to date. Characterization of the EIA antigen obtained from the spleen revealed it to fix complement, have a molecular weight of 27,500 daltons, an S value of 2.1 and be protein in nature with a density of 1.18 to 1.19 g/cm³ by CsCl equilibrium density gradient centrifugation (Norcross, personal communication).

Nakajima et al. (1971) recently reported studies using immunodiffusion tests with a purified EIA virus as antigen. The antigenicity of purified virus was examined by immunodiffusion against sera from horses experimentally infected with EIA virus. Using antigenically different strains of EIA virus, group-specific components of the virus rather than strain-specific ones were considered involved in this reaction. This serological reactivity was lost when antisera from an infected horse was added to the antigen (Nakajima et al., 1971). The precipitation appeared optimally in 0.8% agar gel, 0.85% NaCl and incubation at 32 C for 4 days.

Antigens were prepared as purified virions from CsCl density gradients (Abinanti et al., 1967), and by freezing,
thawing, and shaking of the virus more than 10 times (Altara et al., 1953). Treating with ether resulted in loss of activity (Dalozet, 1939). All three antigenic preparations gave precipitates with positive antisera. Virus in a range of 1.12 to 1.18 g/cm$^3$ buoyant density in CsCl gradients was collected having a S value of 110 and 120 at 20 C (Nakajima et al., 1971).

The following evidence probably indicates the immunodiffusion reaction is specific for EIA: 1) The antigen used was highly purified, concentrated EIA virus. 2) The antigen did not react with preinoculation sera but reacted with sera obtained at some time after the first febrile reaction of EIA. 3) The serological reactivity was lost when the antiserum was added to the antigen. 4) The antigen did not react with sera from horses infected with other viral diseases such as equine rhinopneumonitis virus and cytopathogenic equine orphan virus. 5) The serum of infected horses did not react with suspensions of horse leukocytes, suspensions of cultured horse leukocytes, bovine serum used for the medium, and crude virus material used for preparation of the antigen (Nakajima et al., 1971).

Coat protein or some virion constituent, however, is considered to be involved in the reaction rather than intact virions. The reasons for this are that the virion seemed to be easily disrupted as observed in the EM and the antigen treated with ether or by freezing and thawing did not produce a remarkable change in its reactivity. Furthermore, the
precipitin line was formed as early as 24 hrs in most cases. Thus, small components of the virus are thought to be involved. Since the antigen also reacted with serum from horses infected with different EIA virus strains, the antigen involved is probably an internal component of the virion rather than a surface entity (Nakajima et al., 1971). To date this immunodiffusion test appears the most reliable tested, though its value for detection of the chronically-infected animal is questionable (Nakajima et al., 1971).

C. Virology

1. Viral Propagation

Dreguss and Lombard (1955) attempted to grow EIA virus in cell cultures as well as in several laboratory animals without success. Kono and Kobayashi (1968) reported extensive viral propagation studies using primary horse bone marrow (HBM) and HLC as well as cultures prepared from 16 other equine tissues (Kobayashi and Kono, 1967b). EIA virus propagation was only successful in the HLC and to a limited extent in the HBM (Henson et al., 1969; Kobayashi and Kono, 1967b). Kono and Kobayashi (1967) and Kobayashi et al. (1969) showed that EIA virus in HLC could be serially passed with retention of infectivity.

The HLC used are primary cell cultures in which the cells persist but do not continue in the cell cycle through mitosis. These cell cultures consist of three different cell populations: (1) The granulocytic series of cells which lyse
within the first 2 days in culture; (2) Large mononuclear cells and some lymphocytes which attach to glass and exhibit cytopathic effect (CPE) after EIA viral infection (Moore et al., 1966; (3) Small and medium-sized lymphocytes that are suspended in the culture medium and do not attach to glass. Henson et al. (1969) reported that the percentage of cells that adhere after incubation for 24 hrs represented only 10% of the starting total leukocyte population.

Cytopathogenicity of attached leukocytes has been used as a criterion of infectivity by several workers (Henson et al., 1969; Kobayashi and Kono, 1967a, b; Katsudo and Takaara, 1963; Tokui et al., 1968). The CPE reported consisted of detachment of the leukocytes from the glass surface, and a change from monolayer to suspension culture of the cells. However, CPE as described has not been obtained by other workers who have attempted to establish HLC (Henson, Gorham, Kobayashi, and McGuire, 1969). Nonspecific CPE was described by Kono, Kobayashi, and Fukanaga (1970) in some HLC. A more reliable index of infectivity of cells in cultures has been the demonstration of CF antigen (Henson et al., 1969; Kono and Kobayashi, 1967).

The HLC technique used in many laboratories originated with the work of Kobayashi and Kono (1967a). Heparinized blood was allowed to stand until the erythrocytes sedimented and the leukocytes were washed and placed in culture containers. The medium is usually Medium 199 (M199) plus 20-25% bovine or ovine serum. It has been difficult to maintain
the leukocytes without high serum levels (Henson et al., 1969; Kobayashi and Kono, 1967a). Moore et al. (1966) however, found that the agent could be propagated in leukocytes maintained in M199 plus 0.5% lactalbumin hydrolysate.

It is difficult to establish and maintain HLC in prime condition and the influencing factors are ill-defined. Leukocytes from a good donor horse may be maintained for up to 20 days but such a short longevity severely limits its application to EIA (Nakamura et al., 1968).

Saurino et al., (1966) reported propagation of EIA virus in HeLa cells and KB cells, but these results could not be substantiated by Henson et al., (1969). Moore (personal communication) have established a cell line from peripheral equine leukocytes which appears promising for EIA propagation. Though EIA virus can be propagated in HLC cultures, there is considerable variation and inherent limitations in its use. Thus, a need is still present for a permanent cell line which can be used for EIA viral propagation.

Despite limitations in the HLC system, it has permitted the study of EIA in vitro. Kobayashi and Kono (1967a, b) reported that the EIA virus could be quantitated and titrated in HLC using CPE as an indicator. These workers also showed a parallel with CPE in HLC and CF antibody titer (Kono and Kobayashi, 1967a). The medium from the infected HLC was also used to purify the EIA virus (Nakajima and Obara, 1964; Nakajima et al., 1968).
Kono, Yoshino, and Fukanaga (1970) reported a one-step growth curve of EIA virus in leukocyte cultures and the effect of 5-iodo-deoxyuridine (IUDR) on viral replication. EM was used to observe the tissue cultures. These studies showed a "slow virus" character growth curve. The data indicated that DNA synthesis necessary for virus growth was completed 12 hrs after infection and that formation of mature EIA virus occurred 12-24 hrs after completion of DNA synthesis.

Ley, Burger, McGuire, and Henson (1970) examined leukocyte cultures infected with EIA for production of interferon production although it could be shown that the equine cells are capable of interferon synthesis. Some other proteaceous inhibitory substance is produced which is not affected by actinomycin D (Ley, Burger, and Henson, 1970).

Kono, Kobayashi, and Fukanaga (1970) recently investigated the attenuation of EIA virus by in vitro passage and subsequent immunization of horses. The horses inoculated with a cloned attenuated EIA virus acquired an increase in neutralizing antibody, a slight CF antibody response and no clinical signs of EIA. Such immunized animals resisted challenge with a virulent strain of EIA. Similar results were obtained in recent experiments conducted by Moore et al. (personal communication) using an adjuvant and attenuated EIA virus.

Since the histological changes in chronic cases of EIA are primarily those of the bone marrow, the cultivation
of HbM cells would be of value in the study of EIA (Yamamoto and Konno, 1967). Bone marrow was the only other tissue EIA virus could be shown to persist in other than the HLC as described by Kono and Yokomizo (1969).

2. Electron Microscopic Characterization of EIA Viral Propagation

EIA virus was observed in thin sections of infected HLC by EM (Tajima et al., 1969; Ito et al., 1969). The particles described were not observed in uninfected cultured cells. Appearance of these particles could be prevented by addition of EIA immune serum to the inoculum.

In infected cultures, virions were observed in membrane-bound cytoplasmic vesicles and in extracellular spaces. No free virions were seen in the cytoplasm or nucleoplasm although some particles were closely associated with the external cell surface. The particles were circular to ellipsoidal with a diameter from 80 to 120 nm. They were composed of a dense central nucleoid, viroplasm, and outer coat. Circular nucleoids ranged from 40-60 nm in diameter, and elongated bar-shaped nucleoids had a diameter of 35 by 90 nm. Some particles devoid of a nucleoid were also seen. The viroplasm was a moderately electron-dense zone separating the nucleoid and outer coat. In most of the particles, the nucleoid was separated from viroplasm by a narrow electron-transparent zone. Particles were seen in the process of budding mostly from the cell surface and occasionally from the cytoplasmic vesicles (Tajima et al., 1969).
Henson et al. (1969) recently examined EIA-infected and uninfected HLC for the presence of viral particles. It was easy to demonstrate agents with ultrastructural characteristics of the herpes group of viruses in both the cytoplasm and nucleus of some EIA-inoculated and control cultures. These herpes viruses do not, however, appear related to EIA.

An agent was observed in macrophages in HLC cultures inoculated with EIA virus and not in control cultures. This agent buds from the cell surface, has a relatively translucent core, contains a laminated outer membrane and is approximately 70 nm in diameter. The particle has been consistently observed in infected HLC but the results of horse infectivity have not yet been reported (Henson, personal communication).

Negatively-stained preparations were made from partially-purified EIA virus obtained from infected HLC (Nakajima, Tajima, Tanaka, and Ushimi, 1969). The particles were somewhat pleomorphic, although most of them were spherical and ranged from 90-140 nm in diameter. A well-defined outer envelope was observed in spontaneously disrupted particles, but no organized internal component could be resolved (Nakajima, Tajima, Tanaka, and Ushimi, 1969).

3. Isolation and Purification of EIA Virus

Nakajima et al. (1967) developed a method for virus purification from infected EIA serum. A combined method of ultracentrifugation, DEAE cellulose column chromatography, and sucrose density-gradient centrifugation was used to obtain EIA virus from serum of acutely-infected horses.
Recovery of the virus and protein content were monitored at each step in the purification scheme. In the final virus preparation, the ratio of infective titer to protein concentration was increased 1,000 fold.

Many attempts have been made to purify EIA virus (Nakajima et al., 1967; 1969a, b; Nakajima, Tajima, Tanaka, and Ushimi, 1969) employing ultracentrifugation (Nakajima et al., 1967; 1968), DEAE cellulose column chromatography (Nakajima et al., 1967; 1969a), and CsCl equilibrium density-gradient centrifugation (Nakajima, Tajima, Tanaka, and Ushimi, 1969) of infected culture fluid. The most successful attempt was that reported by Nakajima, Tajima, Tanaka, and Ushimi (1969) using a combination of ultracentrifugation, DEAE cellulose column chromatography and CsCl equilibrium density-gradient centrifugation.

4. Virus Characterization

Physiochemical characteristics reported to date have been based on EIA virus purified from serum. Vallee' and Carre' (Dreguss and Lombard, 1954) in 1904 demonstrated that the EIA agent could be filtered and subsequent work estimated the size of the agent to be between 18-50 nm. Furthermore, it was shown to be ether sensitive (Nakajima and Obara, 1964).

The virus was inactivated by heating at 58-60°C for 1 hr, and by phenol, formalin, ether, and other chemicals (Dreguss and Lombard, 1954; Stein and Gates, 1955; 1952). The agent survived lyophilization and freezing for long periods of time (Dreguss and Lombard, 1954). Partially-purified
virus in CsCl equilibrium density-gradient centrifugation was located between density 1.13 to 1.16 g/cm³ (Nakajima, Tajima, Tanaka, and Ushimi, 1969). This preparation had a CF titer of 1:80 and an infectivity of $10^{6.25} \text{TCID}_{50}/0.5 \text{ml}$. Nucleic acid determinations revealed RNA in fractions purified from infected HLC by Nakajima, Tajima, Tanaka, and Ushimi (1969). Moore (personal communication) reported results of virus characterization by ultrafiltration in which the virus passed a 50 nm filter but was excluded by the 25 nm filter. It was ether and chloroform resistant.

Studies on the one-step growth curve of EIA in the presence of 30 to 50 mcgm IUDR were conducted by Kono, Yoshimo, and Fukanaga (1970).

Virus titers increased rapidly 21 to 36 hrs after infection and gradually until 72 hrs. CPE was detected at 48 hrs postinfection and was complete at 96 hrs after inoculation. The effect of IUDR on the growth of EIA was an initial decrease in growth followed by a slow recovery and was ineffective after 12 hrs postinoculation. The inhibition observed in presence of IUDR was completely reversed by addition of thymidine. Checking blind passage by fuelgen and acridine orange staining gave evidence of DNA. These data indicate that DNA synthesis is necessary for virus growth at least for the first 12 hrs and that formation of mature EIA virions require DNA synthesis. Kono, Yoshimo, and Fukanaga (1970) conclude that this data indicates an indirect involvement in the formation of EIA. Kono, Yoshimo, and Fukanaga
(1970) further consider EIA virus to be an RNA virus based on the observed maturation process.

5. Associated Viruses

Several explanations for the continued presence of EIA virus in diseased horses have been proposed. The agent may be unusual in physical and chemical characteristics; the agent may be replicating intracellularly where it is unavailable for neutralization by antibody; the agent may be infecting and altering the immunocompetent cells; antibody directed against the agent may be unusual and inefficient; or a combination of two or more of these factors may act in concert. The role of these mechanisms in the persistence of EIA virus remains to be clarified, but they are not without precedence in viral disease (Henson et al., 1969).

It has become evident during the past few years that a heterogeneous group of diseases called "chronic viral infections" are relatively common and clinically important to humans and animals (Abinanti, 1967). These conditions are characterized by a prolonged host-virus relationship. Included in this group of viral diseases are Kuru of man, AD of mink, maedi and visna of sheep and scrapie of sheep (Abinanti, 1967; Eklund et al., 1967; Gibbs and Grajdusek, 1965; Gudnadottien and Vaissou, 1965a, b; Thormar et al., 1966). There is a variation among these diseases, i.e. inoculation of the scrapie agent into mice (Eklund et al., 1967) results in slow replication of the agent, principally in the lymphoid tissues, with the disease finally resulting
in death. Virus levels in serum are low in sheep and mice infected with scrapie. Contrarily in AD the agent replicates rapidly in certain mink genotypes with high serum titers which are maintained through the course of the disease. Once initiated AD also progresses slowly but death is inevitable. In both scrapie and AD, the host defense mechanisms are unsuccessful in eliminating the virus from the host (Abinanti, 1967; Eklund et al., 1967).

Equine infectious anemia may be even more complex than these diseases as indicated by the prolonged nature of the host-virus interaction, often lasting many years, and by the recurrence of clinical disease and lesions. The effectiveness of the host defense mechanisms has been abrogated in EIA also, although some defense mechanisms are apparently responsive to the agent (Henson et al., 1969).

a. Equine Herpes Viruses (EHV)

The EHV belong to a large group of viruses widely distributed in nature. These viruses are cubical, enveloped, have DNA, range from 120-180 nm in diameter and consist of approximately 70% protein and 22% lipid (Burrows, 1969).

Tissue culture studies indicate that herpes viruses may be placed into 2 or more less distinct groups on the basis of their growth characteristics (Melnick et al., 1964). One group consists of herpes simplex virus (HSV), infectious bovine rhinotracheitis virus (IBRV) and EHV. They reproduce to moderately high titers and virus is released into fluid phase of the culture. These viruses produce an obvious and
rapid cytopathic effect in cultures prepared from a variety of animals.

In contrast, the other group would include cytomegaloviruses (CMV), herpes zoster and viruses of bovine malignant catarrh have a restricted host range. Many will only grow in cultures from their natural host and generally grow better in fibroblast cell type cultures than in epithelial cell type cultures. Resulting viral titers are low in infected tissue culture media and often the presence of virus is difficult to demonstrate. The areas of cell damage are localized and it may take several weeks to recognize any CPE (Melnick et al., 1964). During the last few years, "slow growing" herpes viruses have been isolated from equine tissue cultures (Husing et al., 1969; Kono and Kobayashi, 1964; Plummer and Waterson, 1963).

Herpes viruses differ from the majority of other DNA viruses (adenoviruses, papova viruses and some pox viruses) in that they are inactivated by ether. The outer membrane of the virus which is acquired by "budding" through one or more host cellular membranes and is thus composed of protein and lipid accounts for the ether sensitivity of this virus (Burrows, 1969).

One of the intriguing questions which remains to be answered for many of the herpes virus infections is whether or not virus remains present in the convalescing animal in a "persistent" infection or as a "latent" infection. That human herpes viruses are retained in the form of latent
infections is well-documented but proof is lacking for similar situations in animals. There is some evidence that CMV remains latently in horses, however (Erasmus, 1969).

b. Equine Cytomegaloviruses

CMV have been isolated from man, mice, guinea pigs, ground squirrels, and African Green monkeys, and are classified as a subgroup of the herpes virus group. CMV differ from other herpes viruses in the following characteristics: (1) Host specificity for viral propagation is restricted; (2) virus replication occurs at a very slow rate and intranuclear inclusion bodies are formed; (3) few infective virions are released into the culture medium although cells may have large numbers present. Basophilic periodic acid schiffs positive intracytoplasmic inclusions can sometimes be seen containing mature virus particles at various stages of degradation and surrounded by dense structures thought to be lysosomes. Many empty virions are also seen in electron micrographs, which might account for the low degree of infectivity which is characteristic of CMV (Erasmus, 1969).

Numerous EHV have been isolated from horses and some have characteristics similar to the CMV group. A number of agents described as cytopathogenic equine orphan viruses, were isolated from kidney and bone marrow of normal horses in Japan (Kono and Kobayashi, 1964). These viruses exhibited a slow growth rate, were resistant to trypsin, and were also serologically distinct from Equine Herpes virus type I (EHV-1). Erasmus (1969) isolated five strains of virus from kidney
cultures during routine tissue culture production. These isolates contained DNA, showed distinct characteristics of CMV and were identified as equine CMV by comparison with EHV-1 and EHV-2 (Erasmus, 1969).

It is difficult to determine where one equine herpes "species" ends and another begins and even harder to decide if EHV types 2, 3 and 4 should be regarded as CMV. The basic question is whether a distinct CMV subgroup of herpes viruses exists phylogenetically, particularly since present criteria for regarding a virus as a CMV are vague. Some investigators feel the criteria must be more rigorously defined before additional viruses are placed in this group (Plummer and Waterson, 1969).

Erasmus (1969) divided EHV into three groups. Group I is represented by the classic EHV (EHV-1, equine rhinopneumonitis) which exhibit the following characteristics: a wide host range, short generation time, high virus titer and short growth cycle. CPE is described as rapid detachment from the glass and cell destruction. The virus is also sensitive to trypsin (Erasmus, 1969).

Group II was represented by HKV-2 and HKV-3 which differ markedly from Group I. They are characterized by restricted in vitro host range, slow growth rate, low infectious titers and resistance to trypsin. There is marked margination of chromatin with many virus particles in infected cells but seldom in extracellular spaces. Basophilic intracytoplasmic inclusion bodies are found occasionally. These
properties are consistent with those described for CMV of other species (Hanshaw, 1969), thus the term "equine cytomegalovirus" was proposed to include HKV-2 and HKV-3 (Erasmus, 1969).

The remaining EHV are put into a third intermediate group. This subdivision is largely based on growth characteristics which may not be totally reliable for classification purposes. Ultimate classification for the EHV must await more detailed examination of the molecular and immunological relationships of the EIA virus and its host cell.
MATERIALS AND METHODS

A. Experimental Animals

1. Horses

Shetland ponies, 2 to 3 years of age, of both sexes as well as young mature standard horses were employed for production of virus. An EIA viral strain, designated Wyoming strain, was used to produce all EIA infections. All animals were kept in an insect free environment. Normal animals were confirmed to be free of EIA by a series of round robins. EIA specimen of bone marrow and EIA serum were produced by the inoculation of serum directly or indirectly from horse number 21 (H-21), a known positive case. This serum was collected on November 15, 1965 and kept at -70 C.

Horse number 17 (H-17) was inoculated with 1 ml of a $10^{-4}$ dilution of H-21 serum on November 16, 1967. After surviving 6 febrile responses this pony was designated a chronic carrier. The chronic status was substantiated by subinoculation of H-17 serum into a normal pony, horse number 59 (H-59).

Normal and acutely infected horses were used as represented in Table 1.

2. Rabbits

Young adult female white New Zealand rabbits were obtained locally and maintained on Purina Rabbit Chow and
Table 1. Data for inoculation of horses for production of EIA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Horse Number</th>
<th>Inoculum</th>
<th>Date of inoculation</th>
<th>Date of 1st febrile response</th>
<th>Date of sacrifice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>H-12</td>
<td>NONE</td>
<td>NONE</td>
<td>NONE</td>
<td>4-15-70</td>
</tr>
<tr>
<td></td>
<td>H-71</td>
<td>NONE</td>
<td>NONE</td>
<td>NONE</td>
<td>7-29-70</td>
</tr>
<tr>
<td>Acutely Infected</td>
<td>H-34</td>
<td>5 ml H-21 serum</td>
<td>4-15-70</td>
<td>4-25-70</td>
<td>4-27-70</td>
</tr>
<tr>
<td></td>
<td>H-28-1</td>
<td>7 ml H-21 serum</td>
<td>5-11-70</td>
<td>5-13-70</td>
<td>5-15-70</td>
</tr>
<tr>
<td></td>
<td>H-64</td>
<td>18 ml H-21 serum</td>
<td>7-01-70</td>
<td>7-07-70</td>
<td>7-14-70</td>
</tr>
<tr>
<td></td>
<td>H-70</td>
<td>3.1 ml of 1:5 dilution EIA purified virus</td>
<td>7-05-70</td>
<td>5-19-70</td>
<td>7-23-70</td>
</tr>
<tr>
<td></td>
<td>H-82</td>
<td>100 ml heparinized whole blood 3rd passage</td>
<td>11-03-70</td>
<td>11-10-70</td>
<td>11-11-70</td>
</tr>
</tbody>
</table>
water ad lib. The rabbits were used for production of anti-EIA serum for immunodiffusion experiments and in the indirect fluorescent antibody test for detection of EIA virus in infected cell cultures.

B. Cell Cultures

1. Media. Eagle's Minimal Essential Medium (MEM) 1640, and Medium 199 (M199) were used to determine the optimal growth conditions of the cells. All equipment used in preparing media was sterilized at 15 psi and 121 C. Media were sterilized through a 0.22 u millipore filter and stored at 4 C. Penicillin-streptomycin, 100 ug/ml, were added to maintain sterility. The standard NaHCO₃ buffer and CO₂ gas system was used in beginning cultures. Later, the pH was maintained using the amino acid, 4-(2-hydroxyl-ethyl)-1-piperazincethane-sulfonic acid (Hepes). It has recently been observed that Hepes buffer maintains the pH better thus stabilizing the micro-environment of the cells more efficiently (Shipman et al., 1968). The result has been an increase in the time of survival for cells in vitro.

To date, it has been extremely rare for any mammalian cell to be grown in vitro in a completely-defined medium. Cells may only be maintained for limited periods without cellular damage in defined medium. It is, therefore, necessary to add some supplement in order to maintain and/or grow cells in vitro for any length of time. A wide variety of supplements are available (peptone, lactalbumin hydrolysate, and
serum), the most common of which is serum. In this case, cells were grown in M199 supplemented with 40% horse serum and buffered with NaHCO₃ with and without Hepes buffer.

Bone marrow cells were obtained aseptically from the femur of horses and a calf. Each sample collected was placed in 100 ml M199-Hepes + 40% horse serum (HS) and 1 ml heparin. The cells were dispersed with a Pasteur pipette and returned to the laboratory for further processing by centrifuging at 800 x g for 15 min. The cell pellet was suspended in fresh medium, plated in plastic tissue culture flasks and incubated at 37 C. Cells were allowed to attach for 24-28 hrs at which time the medium was removed and fresh medium added to the flasks. Cells were kept at 37 C, and fed every 7-10 days. Cells were serially passed by a standard trypsinization technique (Merchant et al., 1964).

2. Organ Explant Cultures. Sections were taken from the spleen and splenic lymph node of H-12. A 1 mm square section was planted in 4 ml of M199-Hepes + 40% HS and M199 + 40% HS in plastic culture flasks and incubated at 37 C. This medium was changed approximately every 4 days.

3. Monolayer Cell Cultures. Samples of the spleen and the splenic lymph node from H-12 were removed and cut into squares. The tissues were trypsinized in 100 ml of trypsin-versene solution (a balanced salt solution containing 0.05% trypsin and 0.2% ethylene diamine tetraacetic acid (EDTA) and while stirring for 2 hr at 23 C. Trypsin was neutralized with 10 ml of HS, the cells centrifuged and the pellet
suspended in either M199 + 40% HS or M199 + Hapes + 40% HS. Four-nl aliquots were placed in plastic flasks and incubated at 37 C. These cells were fed every 24 hrs for the first 2 days and every seventh day thereafter.

4. **Mycoplasma.** Attempts to detect contaminating Mycoplasma in tissue culture was necessary to rule out a source of CPE, cellular transformation, or deoxyribonucleic acids (DNA) in the nucleic acid studies. Mycoplasma agar (GIBCO) was inoculated periodically with a sterile loop of tissue culture medium taken from random tissue culture specimens and from the HS used to feed the cell cultures. The plates were sealed and incubated anaerobically at 37 C and examined on the second, seventh, fourteenth, twenty-first, and thirtieth day after inoculation. Examination of the agar surface for Mycoplasma-like colonies was accomplished in 2 ways: (1) The agar plate was inverted and examined under a dissecting microscope (10 x and 40 x) using oblique light; (2) When any growth was present, a stained agar mount was prepared and examined under oil immersion. A thin layer of Dienes' stain was spread over the surface of 22 mm² cover slips with a sterile cotton swab and allowed to dry.

Colonies to be examined were removed by cutting 1.0 to 1.5 cm² piece of agar with a spatula and placing in on a glass slide with colony facing upward. A stained coverslip was placed over the surface of the agar block, stain side down and the edges were sealed with vaseline to prevent dehydration. Colonies of **Mycoplasma** stain bright blue when mature
and retain the stain in contrast to bacterial colonies which will often stain blue but lose the stain after a short time (Crawford, 1970).

C. Electron Microscopy

1. Preparation of Buffy Coat. One-hundred ml of heparinized blood was collected from normal, chronic, and acutely-infected horses. The acute phase was designated by a febrile peak approximately 10-14 days after inoculation with 1 ml of EIA serum which would produce EIA in normal horses even when diluted 1:6. The blood was centrifuged at 800 \( \times g \) for 4 min, plasma was removed and cold 3% glutaraldehyde was gently layered over the buffy coat and refrigerated at 4 C for 15 min. The glutaraldehyde was aspirated and the WBC pellet removed and placed in a small beaker with fresh cold glutaraldehyde. After rinsing several times in a fixative, the cells were incubated at 4 C for 2-4 hrs. The glutaraldehyde was removed and the pellet minced with a scalpel in fresh glutaraldehyde and washed with Sorenson's buffer with 1% sucrose and stored overnight at 4 C. Cells were post-fixed in 1% osmium tetroxide (OsO\(_4\)) for 1 1/2-2 hrs, rinsed with cold water, and dehydrated in a series of graded alcohols at 4 C. The cells were allowed to reach 23 C in 90% alcohol for about 1 hr when they were washed in 100% alcohol 3 times and 2 times in propylene oxide. They were placed in maraglas:propylene oxide mixture for 30 min. This mixture was replaced with fresh maraglas and specimens were held at 23 C for 1 hr and at 4 C
overnight (Pease, 1964). At this time they were placed in a vacuum oven (15 lb/in$^2$ pressure at 60 C) for 72 hrs in BEMS capsules to remove any bubbles and polymerize the embedding material. Blocks were sectioned with a DuPont diamond knife using an LKB ultratome. Sections were placed on unsupported 400 mesh copper grids and post-stained 1 min in uranyl acetate and 5 min in lead citrate (Pease, 1964) when they were examined using the RCA EMU-3G.

2. Preparation of Tissues. One mm$^3$ sections of the following tissues were taken from an acutely-infected horse: diaphragm, mesenteric, hepatic, and splenic lymph nodes, liver, spleen, pancreas, splenic artery, bone marrow, aortic artery, cerebellum (cortex and interior), necrotic liver, capillary bed and brain stem. The tissues were kept at 4 C in glutaraldehyde until returned to the laboratory when the tissues were embedded using the same embedding procedure described above (Pease, 1964).

3. Preparation of Serum. Infective serum capable of producing EIA when diluted to 10$^{-6}$ was diluted 10$^{-1}$, 10$^{-2}$, 10$^{-3}$, 10$^{-4}$ and 10$^{-5}$ and centrifuged at 3,000 x g for 30 min to clarify the serum. The clear serum was then centrifuged at 40,000 x g in a Sorvall Model RC-2, Centrifuge head SW-34 for 3 hrs to produce a pellet. A drop of suspension from each pellet was placed on a carbon-coated grid and subsequently in the Kinny vacuum evaporator and shadow casted by evaporation of a platinum-carbon pellet from an approximate angle of 15° as recommended by Pease (1964).
4. Thin Sections of Cell Cultures. Bone marrow monolayers of cells were prefixed for 20 min in 3.5% glutaraldehyde in phosphate buffer and pelleted by centrifugation at 2,000 x g for 15 min. The cells were placed in a sucrose wash at 4 °C for 13 hrs and post-fixed 1-2 hrs in 2% OsO₄ in veronal buffer. Cells were dehydrated in cold ethyl alcohol and propylene oxide, embedded in marraglas and sectioned as described above (Feber and Stich, 1969).

D. Isolation and Purification of Virus

1. Gradients. Sucrose gradients were prepared in 0.01 M Tris-HCl buffer, pH 7.2 containing 0.001 M EDTA (McCombs and Rawls, 1968). The concentrations ranged from 10 to 50% in steps of 10% (w/w) with a density range of 1.038 to 1.230 g/ml, pH 6.9-7.1.

Cesium chloride (CsCl) gradients were also prepared in Tris-LDTA buffer. A saturated solution of CsCl in distilled water (22 °C) was mixed with buffer in ratios of 5:8, 4:8, 3:8, 2:8 and 1:8 to give a density range of 1.11 to 1.36 g/ml, pH 7.2.

The gradients were formed by layering equal volumes of these solutions into a standard 5 ml cellulose nitrate tube giving a total of 4 ml per gradient. They were kept at least 4 hrs at 4 °C to allow for diffusion of the layers.

2. Virus Isolation: General. Virus was isolated by three slightly different methods from serum and pooled tissue culture media. The method reported by Nakajima et al. (1969a)
was repeated and results compared with two modified preparative schemes. In the first case, 100 ml of acute BIA serum or 100 ml infected culture fluid were clarified at 7,000 x g for 30 min. The virus was sedimented by centrifugation at 100,000 x g for 60 min. The pellet was suspended in 0.1 M phosphate buffered saline (PBS), pH 7.4 and the suspension centrifuged at low speed. The resultant supernatant fluid was loaded onto diethyl aminoethyl exchange (DEAE) cellulose column (0.98 meq/g) which had been activated with 0.5 M NaCl and equilibrated with PBS before use. After washing the column with 0.1 M PBS the virus was eluted with 1.0 M NaCl. The eluate was concentrated by dialysis. Four ml of the viral concentrate were layered over 1 ml of a saturated solution of CaCl and centrifuged in a 50-50 rotor in the Spincor model L-2 ultracentrifuge at 100,000 x g at 4 C for 22-24 hrs. The sample was fractionated into 0.25-0.4 ml aliquots using a Buchler Instruments Fractionator. The optical densities (OD) at 260 (OD_{260}) and 280 mu (OD_{280}) were determined on each fraction in a Beckman DU-spectrophotometer. The gravimetric method was employed for the determination of the density of each fraction using 10 microliter capillaries. This procedure is presented in Figure 1.

Scheme B was a modification of that used by Nakajima (1969a). A 100-ml sample of serum or conditioned pooled bone marrow culture medium was clarified by centrifugation at 4,000 x g for 30 min. The virus, if present, was pelleted by centrifugation at 100,000 x g for 6 hrs. This pellet and
Virus fluid from cell culture or serum

- centrifuge 7,000 x g, 30 min

  supernatant

  - centrifuge 100,000 x g, 60 min

    supernatant

    - load onto DEAE cellulose column
    - wash with 0.15 M NaCl
    - collect eluate with 1.0 M NaCl
    - concentrate by dialysis
    - lay over saturated CsCl solution
    - centrifuge 100,000 x g, 22 hr
    - fractionate into 0.25 ml aliquots
    - collect virus fraction

Figure 1. Purification Scheme A for Equine Infectious Anemia Virus
each subsequent step was checked by negative stain preparations in the EM for virus-like particles. The pellet was suspended in 3 ml 0.01 M PBS, diluted to 10 ml in distilled water and centrifuged 10,000 x g for 1 hr. The supernatant fluid was centrifuged at 15,700 x g for 1 hr and again at 22,600 x g for 1 hr. This pellet was suspended in 2-3 ml of 0.1 M PBS. One-ml samples were overlaid on a 10-40% CsCl gradient and centrifuged 100,000 x g at 4 C for 22-48 hrs. After centrifugation, the gradient was fractionated into 0.25-0.30 ml aliquots using a Buchler Instruments Density Fractionator. The OD_{260} and OD_{280} and the density of each aliquot were determined. The OD peak with a density from 1.12-1.16 g/cm^3 was collected and frozen as purified EIA virus. This scheme is represented in Figure 2.

Scheme C was identical to Scheme B for the first three steps. The virus-like particles were pelleted from the sample at 100,000 x g for 6 hrs and suspended in 3.0 ml 0.01 M PBS. One to two ml of this pellet suspension was overlaid onto a 10-50% sucrose density gradient and centrifuged at 100,000 x g for 18-24 hrs at 4 C. The sample was fractionated into 0.25 ml aliquots and the OD_{260} and OD_{280} determined for each aliquot in the Beckman DB Spectrophotometer. The 2-3 peak fractions were overlaid onto a second 10-40% sucrose density gradient and centrifuged at 100,000 x g at 4 C in a SW-39 rotor for 18-22 hrs. This sample was fractionated into 0.25 ml aliquots and the OD_{260} and OD_{280} and density of each aliquot determined. The absorbing
100 ml acute EIA serum
or
100 ml infected culture fluid

100,000 x g, 30 min

<table>
<thead>
<tr>
<th>supernatant</th>
<th>pellet</th>
</tr>
</thead>
</table>

100,000 x g, 6 hr

<table>
<thead>
<tr>
<th>supernatant</th>
<th>pellet</th>
</tr>
</thead>
</table>

suspend in 3 ml 0.01 M PBS
dilute to 10 ml in sterile distilled water.
Centrifuge 10,000 x g, 1 hr

<table>
<thead>
<tr>
<th>supernatant</th>
<th>pellet</th>
</tr>
</thead>
</table>

15,000 x g, 1 hr

<table>
<thead>
<tr>
<th>supernatant</th>
<th>pellet</th>
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22,000 x g, 1 hr

<table>
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<tr>
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<th>pellet</th>
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lay over CsCl gradient
Centrifuge 100,000 x g, 22 hr

<table>
<thead>
<tr>
<th>supernatant</th>
<th>pellet</th>
</tr>
</thead>
</table>

fractionate into 0.25 ml aliquots
collect virus fraction and freeze at -20 C

Figure 2. Scheme B for isolation and purification of EIA virus from serum or tissue culture fluid.
Figure 3. Scheme C for the isolation and purification of EIA virus from infected cell culture fluid and serum.
fractions with a density of 1.12-1.16 g/cm³ were considered as purified EIA virus and were frozen at -70 °C. This scheme is represented in Figure 3.

3. Density Determination. One ml of purified virus preparation was laid over a 4 ml 15% CsCl cushion and centrifuged with a control sample of Escherichia coli DNA in the Spinco Model E SW-39 rotor at 110,000 x g at 4 °C for 22 hrs. The gradient was fractionated into 0.25 ml aliquots and the OD₂₆₀ and OD₂₈₀ determined. The density of each aliquot was calculated by a gravimetric method using 10 ml capillaries and Cahn Gram Electrobalance Number 1500 (Nakajima, 1969a). Optical density determinations were also performed using the Beckman DB Spectrophotometer.

E. Cytochemistry

Leighton tube coverslips of bone marrow monolayers were fixed in formalin-acetic acid alcohol for 1 hr. Cells were stained with methylene blue by standard procedures (Merchant, 1964). Coverslips of cells were also stained with fuelgen stain by the standard technique (Merchant, 1964). Slides were mounted with Permount and examined with a Leitz 250 fluorescence microscope.

F. Nucleic Acid Extraction

Several methods of nucleic acid extraction were used to elucidate nucleic acid species and optimum recovery.

1. DNA Extraction by Freifelder's Method (1966). Purified virus was obtained as described and 5 ml the suspension
used for each DNA extraction. To the virus suspension was added 6 M perchloric acid (HClO₄) in 10⁻² M EDTA to a final concentration of 5 M KClO₄, pH 7.0. The protein and nucleic acid was precipitated in an excess of 95% ethanol. The precipitate was suspended and dissolved in dilute saline-citrate (SSC). Protein was removed with chloroform:isooctyl alcohol (24:1) until no more protein was seen at the interface. The nucleic acid was precipitated again with 95% ethanol and allowed to set overnight at 4°C. The precipitate was dissolved in SSC and stored until assayed.

2. DNA Extraction by Swetty's Method (1969). A 1 ml suspension of purified virus from H-21 EIA serum was mixed with 1 ml pronase (50 mg/ml) and incubated at 37°C for 1 hr. Two ml of 80% phenol (9 parts phenol: 1 part 2 M trichloroacetic acid) were added and shaken 15 min and centrifuged 8,000 x g for 15 min. The interface was extracted two times with 1 ml 0.05 M Tris-Cl (pH 8.2) and 0.5 ml 1.0 M NaCl. Aqueous phases were pooled and extracted with 80% phenol, shaken 15 min and centrifuged 8,000 x g for 15 min. The aqueous phase was extracted four times with cold petroleum ether to remove the remaining phenol. Ether was eliminated with a stream of nitrogen. DNA was precipitated with cold ethanol and centrifuged at 15,000 x g for 15 min. It was collected in 0.1 ml SSC and measured colorimetrically by the diphenyl-anine test.

3. DNA Extraction by Marmur's Method (1961). Purified EIA virus was suspended in 2.5 ml EDTA-versene (pH 8.0)
and 0.25 ml of 25% sodium lauryl sulfate solution (SLS) followed by 10 min incubation in a water bath at 60 C. To the viscous preparation was added enough perchloric acid (final concentration 1 M) and an equal volume of a mixture of chloroform and isoamyl alcohol (24:1, v/v). The sample was shaken for 30 minutes and centrifuged at 2,000 x g for 10 min at 4 C. The upper aqueous layer was transferred to a clean tube using a wide-bore pipette to reduce damage due to shear forces. Deproteinizations with chloroform-isoamyl alcohol were repeated until the interfacial precipitate had disappeared. Two volumes of 95% ethanol were layered carefully on top of the final aqueous layer which precipitated the DNA. It was wound on a glass rod and dissolved in 2 to 5 ml of 0.1 x SSC. The SSC concentration was adjusted to that of 1 x SSC with 10 x SSC which was followed by incubation for 30 min at 37 C with RNase (50 ug/ml). The deproteinization steps were repeated with the DNA finally being dissolved in a volume of 1-2 ml of 0.1 x SSC. When enough yield was obtained, the DNA was added to 4 ml of 40% (1.42 g/cm³) CsCl and centrifuged in a SW-39 rotor at 60,000 x g for 12 to 16 hrs at 4 C. The pellet was resuspended in 2 ml SSC. Isopropyl alcohol (0.54 volume) was added dropwise while spooling the DNA on a glass rod. DNA was stored dissolved in SSC along with a few drops of chloroform as a preservative.

4. Preparation of DNA for Electron Microscopy. DNA from EIA virus was prepared for electron microscopy by a procedure modified from the protein film technique of Kleinschmidt (1963).
One volume of DNA at 2 ug/ml in 1 M ammonium acetate
\((\text{NH}_4\text{C}_2\text{H}_3\text{O}_2)\) was mixed with one-tenth volume of 0.1% (w/v)
cytochrome C; 0.002 ml of the mixture was spread onto a 5 cm²
surface of 0.15 M \(\text{NH}_4\text{C}_2\text{H}_3\text{O}_2\). The resulting film was pricked
with carbon-parlodion-coated grids and dehydrated in 95%
ethanol for 30 seconds (Anderson, 1970). Preparations were
then shadowed at a 10° angle with a carbon-platinum rod from
2 directions. DNA preparations were observed using the
RCA EMU-3C. Micrographs were taken at a magnification of
30,000 to 40,000 x.

5. Ribonucleic Acid (RNA). A modification of the method
of Martin (1969) was used in an attempt to isolate RNA from
the virus preparations. Two ml of purified virus preparation
was mixed with 4 volumes of buffer containing 0.01 M sodium
acetate \((\text{NaC}_2\text{H}_3\text{O}_2)\) (pH 5.3), 0.1 M NaCl, 1 mg/ml bentonite,
and 0.5% SLS. Two volumes of water-saturated phenol were
added and the contents shaken 15 min at 23 C and subsequently
for 15 min in a waterbath at 60 C. Following proteinization
with phenol, the preparation was centrifuged at 16,000 x g
for 20 min and the aqueous layer removed and re-extracted
with phenol at 60 C. After a second centrifugation, the
aqueous phase was precipitated with 2 volumes of cold absolute
ethanol. Analytically pure DNAase was added to a final con­
centration of 50 ug/ml in the presence of 0.002 M MgCl₂ and
0.01 M \(\text{NaC}_2\text{H}_3\text{O}_2\) (pH 5.3). The mixture was incubated at 23 C
for 6 hrs and incubated for 18 hrs at 23 C with 50 ug/ml of
pronase per ml preparation. The pronase had been self-digested
at 37 C for 2 hrs in 0.05 M Tris buffer (pH 8.0). This was followed by a third deproteinization with phenol and centrifugation and precipitation of the aqueous phase with 2 volumes of cold ethanol. If a RNA precipitate was detected it was then applied to a Sephadex G-100 column equilibrated with 0.1 M NaCl and the RNA in the excluded volume collected. A final precipitation of the RNA with ethanol was done and the RNA was stored at 4 C.

6. DNA Determination. Determination of DNA was made colorimetrically by the modified method of the diphenylamine (DPA) reaction (Shatkin, 1969). Stock solutions of calf thymus DNA (Boehringer Corporation) and Salmon Sperm DNA (Calbiochem) were prepared for standards.

Two ml of DPA reagent was added to 1 ml of acid extract (0.5 N HClO₄) of the DNA extract. The tubes were covered with parafilm and incubated 20 hrs at 23 C. The OD₂₆₀ was read against a blank containing only 0.5 N HClO₄.

7. RNA Determination. The Dnal reaction more commonly called the Orcinol Test by Shatkin (1969) was used to test for RNA. Standard RNA solutions were made with 0.5 mg/ml of yeast RNA in water and diluted as necessary.

One ml of freshly prepared reagent was added to 1 ml of standard RNA solution or unknown material. Tubes were then placed in a boiling water bath for 45 min, cooled and the OD₆₇₀ read using a water blank.
G. Sedimentation Equilibrium Centrifugation

A stock solution of CsCl was prepared by dissolving 13 gm in 7 ml of 0.02 M Tris buffer, pH 8.5 (Mandel et al., 1968). The mixture to be centrifuged consisted of 0.84 ml of CsCl stock solution, 0.01 M of Escherichia coli DNA solution (about 50 ug/ml), and 0.04 ml extracted DNA. The density was adjusted to 1.71 g cm\(^{-3}\) \((n = 1.400)\) with water or CsCl stock solution using the following relationship:

\[ P_{25^\circ C} = 10.8601 n_D 25^\circ C - 13.4974 \]

Approximately 0.70 ml of the sample was placed in an ultracentrifuge cell with a 12 mm Kel-F centerpiece. The cell was seated in an An-D rotor and placed in a Beckman Model E Analytical Ultracentrifuge equipped with UV optics. The cell was centrifuged at 44,000 rpm at 25\(^\circ\)C until equilibrium was attained (approximately 20 hrs).

Buoyant density was calculated using the following equation (Sueoka, 1961)

\[ P = P_0 + 4.2 w^2 (r^2 - r_0^2) \times 10^{-10} \text{ g cm}^{-3} \]

The \(P_0\) was taken as 1.724 g cm\(^{-3}\), while \(r\) and \(r_0\) was the distance from the center of rotation of the unknown and reference DNA peaks, respectively. The angular speed, \(w\), is expressed in radians/sec. For a speed of 44,000 rpm \(4.2w^2 \times 10^{-10}\) equals approximately 0.0090.

The mole fraction \(GC\) of native DNA was calculated from the linear relation of Schildkraut et al. (1962)

\[ GC = \frac{P - 1.660}{0.098} \]
Photographs were taken and tracings of the film were made with a densitometer. The buoyant density of the DNA was calculated from distance measured on the tracings.

H. Immunological Characterization

1. Immunodiffusion

Gel diffusion plates were prepared by coating 4" x 5" glass slides with 4 ml 1% purified agar and allowed to dry to a thin film. This was then coated with 15 ml of 1% agar and allowed to solidify. The wells were 2 mm in diameter and 4 circumferential wells were placed at a distance of 4 or 8 mm from the center well. The central well was filled with purified virus and the peripheral wells with serum. The plate was incubated in a moist chamber at 22 C for 4 days. When the precipitin lines formed, the plate was removed from the incubator and stored at 4 C (Nakajima, personal communication).

Prior to staining, the plates were washed for 3 days in normal saline and rinsed for a few hours to remove excess protein. The slide was covered with a piece of wet filter paper and air dried. The precipitin arcs were stained by immersing the plates for 10 min in 0.2% acid fuchsin in a mixture of 10% acetic acid and 50% methyl alcohol. Subsequently, it was washed with 2 or 3 changes of acetic acid-methyl alcohol solution during a period of 15 to 20 min (Carpenter, 1965).
2. Fluorescent Antibody

Thirty to 50 ml of blood were removed from normal rabbits as preimmunization control serum by a cardiac puncture. Purified virions were prepared, dialyzed free of excess CsCl or sucrose and 30-150 ug injected IV into a rabbit. A sample of the normal horse serum pellet from centrifugation at 100,000 x g was inoculated into a rabbit to produce control antiserum. Bleedings were taken 3, 6, and 12 days post immunization and sera frozen and tested later for specific antibodies to the whole virion by an agar-gel precipitin test. Two weeks later, 30-150 ug intact virus was emulsified in an equal volume of complete Freund's adjuvant and injected into each of 4 rabbit foot pads. Three to 4 weeks later a trial bleeding was taken and if antibody was present the animal was bled at 3-4 day intervals until 100-200 ml serum were obtained (Shatkin, 1969). Antiserum was placed in tubes of 10 ml each and stored at -20 C. Before use the antiserum was absorbed twice with beef liver powder to reduce nonspecific fluorescence and clarified by centrifugation at 20,000 x g.

Bone marrow from acutely-infected EIA ponies, acute bone marrow (ABM) from normal ponies, normal bone marrow (NBM), and calf bone marrow (CBM) were grown to confluent monolayers in Leighton tubes on 6 x 22 mm coverslips. Duplicate samples were run in all cultures. One was preadsorbed 1-2 hrs with purified EIA virus diluted 1:10 to 1:100 in tissue culture medium and sterilized by millipore filter.
**a. Indirect Fluorescent Antibody Procedure.** The Leighton tube coverslips with a monolayer of bone marrow cells were removed and allowed to air dry 15-30 min. They were subsequently fixed in acetone for 10 min at 23 C. After fixation, the slides were rinsed in fluorescent antibody (FA) buffer and placed in a moist chamber. One drop of antisera was placed on the cells and the chamber placed at 37 C for 30 min. The slides were washed 3 times with PBS for 10 min and incubated with anti-rabbit conjugated florescent Fluorescenc Isothio-cyanate (FITC) (Commercial preparation in a goat gamma globulin - Difco) for 30 min.

The slides were washed again 3 times and counterstained with rhodamine B (RBA) (diluted 1:5) for 30 min in a humid chamber at 37 C. The slides were rinsed in FA buffer for another 2-3 minutes and then mounted in phosphate-buffered glycerol and examined. Slides were viewed with a Leitz fluorescence microscope.

The bone marrow (BM) preparations were observed for:

1) Autofluorescence before application of antisera or conjugate.

2) Nonspecific fluorescence after application of specific antisera or conjugate.

3) Specific fluorescence after application of specific antisera and conjugate.

4) Masking of nonspecific fluorescence after counterstaining with RBA.
To test the specificity of the antisera, the following preparations were stained: CBM, NEM, ABM, and preabsorbed BM. Preparations of these cells were also reacted with antisera prepared in rabbits and normal horse blood. The results of the immunofluorescence (IF) were interpreted as positive or negative according to the number of cells fluorescing and the intensity of fluorescence (Goldman et al., 1968; Kottaridis and Luginbuhl, 1968; Purchase, 1969).

b. Direct Fluorescent Antibody Procedure. Identical cultures used in the indirect procedure were used in a direct FA test for detection of EHV. FITC conjugated anti-EHV serum was obtained from Dr. Kemeny at NAMD for detection of EHV antigen in these BM cells. The presence of EHV in tissue culture from horses or those tissue cultures fed with horse serum is well documented (Holborow and Johnson, 1967; Goldman et al., 1968). Bone marrow cultures were removed, air dried, fixed in acetone and flooded with 1:5 dilution of conjugated anti-EHV antiserum. Slides were incubated in a moist chamber at 37 C for 1 hr, rinsed 3 times in FA buffered glycerol and observed.
RESULTS AND DISCUSSION

A. Tissue Culture

Cells obtained from horse and CBM, spleen, and splenic lymph nodes (SLN) were successfully grown in vitro. Attempts to cultivate RBC in various media supplemented with varying amounts of serum or peptone revealed M199 + 40% normal horse serum (NHS) to be the best. The length of time that these cells could be maintained was increased by the addition of Hapes buffer to the medium. It was also noted that the cells as illustrated in Figure 4 were grown in Hapes-buffered medium grew to a more confluent monolayer. In contrast, cells cultured in M199 + 40% NHS without Hapes buffer were sparse as illustrated in Figure 5. Splenic and SLN cell cultures could only be maintained for a few weeks as represented in Table 2. No CPE was observed in these cell cultures nor could any viral-like particles be detected in thin-sections examined by EM.

Splenic and SLN organ explant cultures were maintained in M199-Hapes + 40% NHS. These cultures survived for a few months as indicated in Table 2. Attempts to detect virus in the media from the cultures and by examining thin-sections of the cells by EM did not reveal viral-like particles. Examination of the cultures for Mycoplasma was also negative.
Table 2. Survival time of equine explant cultures in vitro

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<th>Animal Number</th>
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Set Up 5/15/70

| H-12          | SM               | S-1            | M199+HS\(^1\)+He\(^2\) | 0                  | 9                       |
|               |                  | S-8            | M199+HS\(^1\)          |                    |                         |
|               |                  | 130-138        | M199+HS\(^1\)+He\(^2\) | 0                  | 9                       |
|               |                  | 140-149        | M199+HS\(^1\)+He\(^2\) | 0                  | 9                       |
|               |                  | 150-158        | M199+HS\(^1\)+He\(^2\) | 0                  | 9                       |

Set Up 11/16/70

| H-12          | Spleen           | 31             | M199+HS\(^1\)+He\(^2\) | 0                  | 18                      |
|               |                  | SLN            | M199+HS\(^1\)+He\(^2\) | 1                  | 48                      |

Set Up 4/15/76

1. 40% HS
2. He = Hepes 3.3 gm/l
3. SLN - Splenic lymph node
4. SM - Splenic monolayer
Table 3. Survival time of bone marrow cultures in vitro

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Set Up 3/5/70

Set Up 3/15/70

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Set Up 7/29/70

Set Up 10/29/70

Set Up 11/13/70
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Set Up 3/7/70

1 40% HS
2 NBM – Bone marrow from a normal horse
3 He = Hepes 3.3 gm/l
4 ABM – Bone marrow from a horse acutely infected with EIA
5 CBM – Bone marrow from a calf infected with Anaplasmosis
Single populations of primary fibroblastic cells from bone marrow have been found to proliferate reproducibly for 2 to 10 months if maintained in logarithmic growth. During this time cells remained uniform in appearance, and no signs of deterioration of the culture as a whole were observed. Later, mitotic activity decreased, and degenerative changes occurred, with ragged cells, aberrant nuclei, and accumulation of debris at cell surfaces.

Hayflick and Moorhead (1961) were unable to reverse the degenerative trends in primary cell cultures by changes in media, subcultivation, or pooling of populations. They did, however, rule out the direct action of toxic factors in the external medium as the reason for decline and loss of cell strains at this stage. Young strains were able to proliferate vigorously in the same pool of medium in which older strains progressively deteriorated. This experiment was repeated using young fresh BMC in media from an ABM culture in its tenth passage and results were similar. Thus deterioration of cells is thought to be an intrinsic sequence of change.

Bone marrow was the best source of cells for in vitro studies used in these studies. The cells grew more rapidly initially when taken from animals acutely-infected with EIA. Longevity of ABM, NBM, and CBM cells are represented in Table 3. In general, these cells subsisted several months longer than cultures of spleen or SLN.

The normal BMC and CBM cultures could be subcultured and maintained for a longer period of time than the ABM
cultures although the ABM had a faster initial growth rate. Figures 6 and 7 represent a monolayer of CBM and NBM, respectively. No viral-like particles could be isolated from pooled media from NBM or CBM cultures. These cell cultures did not form the "foci" as early as observed in ABM cultures. The CBM or NBM cells began to round up only at the time of transfer into fresh medium in which the population of cells exceeded the nutrients available or if the cells were not fed every 7 to 10 days. In contrast the ABM cultures began to form foci before a confluent monolayer was formed. The initial stages of foci development are illustrated in Figure 8 and fully developed foci are shown in Figure 9. Media pooled from ABM cultures at time of feeding and transfer were used for virus isolation and purification. Virus particles with a density of 1.146 g/cm$^3$ were isolated by equilibrium density gradient centrifugation. Subsequent inoculation of this material into a normal pony produced a febrile response in 18 days which was indicative of EIA. That the presence of EIA virus in these ABM cultures could be responsible for the foci formation was further evaluated by adsorption of NBM, CBM and ABM with purified virus. No demonstrable changes were detected in NBM 2 days after inoculation of purified virus onto the cells diluted in M199 as illustrated in Figure 10. The virus inoculum was the same as prepared from H-21 serum and which on subsequent inoculation produced an acute infection of EIA. ABM adsorbed with purified EIA virus from H-21 serum produced no detectable changes as illustrated in Figure 11. Some
Figure 4. Acute bone marrow monolayer grown in M199 + 40% horse serum and Hepes buffer. Mag. 450 x.
Figure 5. Acute bone marrow monolayer maintained in M199 + 40% horse serum with bicarbonate buffer illustrating sparseness of cells without Hepes buffer. Mag. 450 x.
Figure 6. Normal bone marrow monolayer grown in M199 + 40% horse serum and Hepes buffer. This monolayer is more confluent than observed in acute bone marrow cultures. Magn. 450 x.
Figure 7. Calf bone marrow monolayer grown in M199 + 40% horse serum and Hepes buffer. In the tenth passage this monolayer is more confluent than younger acute bone marrow cultures. Mag. 225 x.
Figure 8. Acute bone marrow cells beginning to retract from a monolayer into foci. Mag. 450 x.
Figure 9. Acute bone marrow cells with foci formation established. Mag. 450 x.
Figure 10. Normal bone marrow monolayer of cells 1 day after inoculation with purified virus. No CPE can be detected. Mag. 900 x.
Figure 11. Acute bone marrow cells in the fourth passage after maintenance in M199 + 40% H-21 (EIA positive) serum and Hepes buffer. No CPE was observed after the addition of infectious serum. Mag. 900 x.
Figure 12. Acute bone marrow monolayer 4 days after adsorbing with purified virus from EIA infected serum. No CPE is detected although the cells can be observed to begin formation of foci. Mag. 900 x.
retraction of cells were observed in ABM without addition of isolated virus. ABM cultures fed several months with H-21 EIA positive serum were not observed to be any different than ABM cells fed with NHS supplemented medium as illustrated in Figure 12.

Thus degeneration of cultures was not a function of viral-induced CPE. The slight cellular morphological alterations detected were studied more closely by EM.

B. Electron Microscopic Studies

Thin sections of leukocytes from the buffy coat were usually found to be free of virus. Occasionally 45 nm-dense particles were observed in the nucleus of these cells. These particles might represent cores of incomplete virion—perhaps EIA or some other equine virus. The cells containing such particles were rare and probably indicated a low titer of virus present. An attempt to increase the number of infective particles was made by inoculating cell cultures with EIA virus isolated and purified from infected serum.

Virus-like particles isolated from EIA serum and prepared by negative-staining and shadowing were 45 to 90 nm in diameter as illustrated in Figure 13. Both particles were present in the same CsCl gradient fraction suggesting they were of close molecular weights. These may represent incomplete and complete enveloped EIA virus or two different viruses. These results did, however, establish a size range for the virus present in EIA serum.
Particles of the same size range were also observed in BMC cells by EM. This virus appeared to have a low titer as suggested by the low number of cells containing viral particles. This virus has only been shown to grow in HBM and HLC suggesting host specificity. Cells which were infected displayed characteristics commonly found in equine cells infected with a herpes-type II virus often referred to as equine CMV. This sequence of events observed in common with CMV is in contrast to observations for EIA made by Ito et al. (1969). Ito et al. (1969) studied HLC infected with EIA virus as opposed to BMC as used in this investigation. In the HLC studies, three days after infection, approximately half the cells remained attached to glass. EM examination of thin sections at this stage revealed EIA virus particles in the cytoplasm and its related membranous systems. Further multiplication of the particles are inhibited by adding horse serum containing EIA neutralizing antibodies to culture medium (Kono, 1969).

The EIA virus preparation observed by Henson et al. (1969) (type II) was quite different from the virus reported by Nakajima, Tajima, Tanaka and Ushimi (1969), Ito et al. (1969) and Tajima et al. (1969) (EIA type I). The data with BMC infected with EIA virus are more consistent with that reported by Henson et al. (1969) who also reported 2 distinguishable particles. Previous descriptions were made on virus found in leukocyte cultures as opposed to this work with BMC.
The HLC virus particles described by Ito et al. (1969) had a size range of 80 to 120 nm in diameter and contained an electron dense nucleoid separated from a well-defined limiting membrane by a zone of viroplasm which is electron opaque. The nucleoid was 40-65 nm in diameter, somewhat pleomorphic to spherical. The virions were observed mainly in the lumina of cytoplasmic vacuoles, on the free surface and in intracellular space of infected HLC both in HLC described by Ito (1969) and in this data with HBC. The greatest concentration of particles was found in the narrow lumina of small cytoplasmic vacuoles as illustrated in Figure 20. No crystallike arrangements were found, nor were virions found in the nucleus (Ito et al., 1969).

Tajima et al. (1969) reported the virus particles observed in infected cultures to be in membrane-bound cytoplasmic vesicles or vacuoles or in intracellular spaces. Particles were not observed to be free within the cytoplasm or nucleoplasm. Extracellular particles were seen free and in close association with external surface of the cell membrane, and often relatively large numbers of virions could be seen attached to cellular debris. These particles were circular or ellipsoidal and varied in size ranging from 80 to 120 nm in diameter. They were composed of a dense central nucleoid, viroplasm and outer coat. The nucleoids were roughly circular within a size range from 40 to 60 nm. Elongated bar-shaped nucleoids, with a diameter of about 35 to 90 nm were frequently seen (Tajima et al., 1969). It is observed that these
characteristics are strikingly similar to herpes viruses, particularly the bar-shaped electron dense nucleoids which also fit the size range observed for EBV (Melnick et al., 1964).

Particles observed by Tajima et al. (1969) in the cytoplasmic vesicles or vacuoles were morphologically identical to those observed extracellularly in ABM as illustrated in Figure 15. These virus-containing vesicles varied in size and shape and were set-off from the surrounding cytoplasm as seen in ABM by a membrane as illustrated in Figures 16 and 19. Cellular debris was often found inside these vacuoles which Tajima (1969) suggested represented a phagocytic activity of the cells. This might just as well represent coat material removed in uncoating of virus after entry by lysosomal enzymes. One observes a similar process in replication of canine herpes-viruses as described by Nii et al. (1968).

EBN viral particles have been observed budding from cell surface (Tajima, 1969) and from cytoplasmic vesicles (Ito et al., 1969). Tajima (1969) observed that the EBN virus replicates primarily by a process of budding from the plasma membrane of infected cells. Virus particles within membrane-bound cytoplasmic vesicles or vacuoles are those phagocytized by membrane invagination of cells after being liberated from other cells into the medium. He supported this observation with the fact that most of the virions were seen budding at the plasma membrane and that only a limited number were budding from cytoplasmic vacuoles. In contrast,
the EIA virus observed in ABM cells were seen to bud more from cytoplasmic vacuoles than from the cell surface. Such a budding process is illustrated in Figure 16.

The EIA virus as described by Henson et al. (1969) agrees with this data from infected BM but is markedly distinct from that described by Ito et al. (1969). The EIA virus described by Henson et al. (1969) has a relatively translucent core, contains a laminated outer membrane and is approximately 70 nm in diameter. This virus buds from the cell surface and was observed in macrophages in HLC infected with EIA virus. This agent was not detected in control cultures.

Another virus was observed in HLC infected with EIA virus, however. This second virus has ultrastructural characteristics of herpes group of viruses. This second agent was observed in the cytoplasm and nucleus of EIA-inoculated and control cultures. The pathology of EIA does not seem to involve this agent, however (Henson et al., 1969).

EM examination of ABM cells revealed the presence of the 2 morphologically distinct viruses described previously in HLC. EIA viruses type 1 as described originally by Nakajima, Tajima, Tanaka, and Ushimi (1969), Ito et al. (1969) in EIA infected HLC were observed to be free in extracellular space along with cellular debris as illustrated in Figure 17. These virus particles measure 120 nm in diameter and contain an electron dense nucleoid which is 45 nm in diameter. This virus was observed budding from a cytoplasmic vacuole and
received an outer coat in the process. The budding process is represented in Figure 16. The complete mature virion type I measures 100 nm in diameter and resembles EHV described by others (Kleinick et al., 1964). A second virus was observed with a translucent core and laminated outer membrane illustrated in Figure 16 by a second arrow. This virus was not observed in control cultures and measures 70 nm in diameter in agreement with the particles by Henson et al. (1969).

Electron dense nucleoids 15 nm in diameter were occasionally observed in the nucleoplasm of infected ABM cells. Rarely, a nucleus of an infected cell was observed with a concentration of electron dense cores and aberrant capsids as illustrated in Figure 17. The nucleoids measured 40-60 nm in diameter and the complete particles were 80-120 nm in diameter. This observation closely resembles that reported for the canine herpes virus by Nii et al. (1968). Similar particles were not observed in control cells; however, many more ABM cells were examined for virus than were control cells.

Margination of nuclear chromatin in ABM cells was often observed, which is a common characteristic of the replicative events of EHV. This characteristic is illustrated in Figure 18 and viral-like particles (Type II) were detected in the cytoplasm. These virions measured 60-70 nm and were morphologically similar to the EIA agent reported by Henson et al. (1969).
Inoculation of ABM cells with purified EIA virus from infected serum by Scheme I increased the concentration of detectable virus in cells examined by EM. As illustrated in Figure 26 both viral types are observed in the cytoplasm. The herpes-like type I particle measured 80-120 nm and the type II viral-like particles measured 60 to 75 nm in diameter. Despite a detectable increase in intracellular virus concentration as observed by EM no macroscopic effect was noted in the cell cultures prior to fixation for embedding.

The herpes-like particles also reported to be the EIA virus by Nakajima, Tajima, Tanaka, and Ushini (1969) could possibly be an equine CMV. The equine CMV are characterized as having a slow growth rate with few infective particles liberated into the growth medium despite large numbers inside the cells. CMV displays a restricted host range and will only grow in cells from its natural host. Basophilic, PAS-positive intracytoplasmic inclusions are often found and sometimes nuclear inclusions. The cytoplasmic vesicles are observed to contain mature CMV in different stages of degradation and dense structures thought to be lysosomes (Erasmus, 1969). This feature could represent a protective mechanism of the host cell and offer a possible explanation for the low infectivity titer of the growth medium. The CMV is also resistant to trypsin in contrast to other EIV. The characteristics observed for the EIA-associated virus in ABM cells are in common with those described for CMV. However, further work needs to be completed before EIA can be classified as a CMV.
It has been suggested by Ito et al. (1969) and others that the replicative events observed by EIA are similar to the leukemia virus group. It has thus been presumed to be an RNA virus. It should be noted that ED was first thought to be a leukosis virus but later was shown to be a herpes virus.

The typical herpes type I viruses, i.e. herpes simplex virus, are not usually found associated with anemias (Nii et al., 1968). However, Armstrong et al. (1970) reported CMV was found associated with the RBC as well as leukocytes in the blood of patients with cytomegaloviremia. Since the mechanisms involved in EIA are not known, it is possible that at least part of the pathology of the anemia could be viral as well as immunological.

No viral particles or morphological characteristics which suggest viral infection were observed in RBC by EM as illustrated in Figure 21.

These data suggest several possibilities:

1) That two distinguishable viruses have been incriminated for EIA and that only one is responsible for the disease and the other is insignificant.

2) That the pathology of EIA is a consequence of two viral agents acting in concert.

3) That these two agents act independently but both are required to cause an acute infection of EIA.

4) That one virus has two morphological forms.
Figure 13. Electron micrograph of a shadowed preparation of purified EIA virus from H-21 serum. Mag. 34,500 x.
Figure 14. Electron micrograph of a normal bone marrow cell prepared by embedding and thin sectioning for observation. No viral like particles or other characteristics of viral infection can be detected. Mag. 25,000 x.
Figure 15. Electron micrograph of a thin section of an acute bone marrow cell. Herpes like virus particles (I) measuring 100-120 nm in diameter are present in extracellular space associated with cellular debris (D). This type I particle is analogous to the EIA virus described by Ito et al. (1969) and Nakajima, Tanaka, and Ushimi (1969). Mag. 30,000 x.
Figure 16. Electron micrograph of herpes-like virus particles (EIA type I) budding from a cytoplasmic vacuole in an acute bone marrow cell. The mature enveloped virion measured 100 nm in diameter. Type II particles 70 nm in diameter are labeled II. Mag. 30,000 x.
Figure 17. Electron micrograph of the nucleus of an acute bone marrow cell. The nuclear region contains a concentration of 40-60 nm nucleoids (N) and aberrant capsids (Ab). The mature coated virion measures 120 nm in diameter indicated by the arrows and resembles other herpes-like viruses. Mag. 42,500 x.
Figure 18. Electron micrograph of an acute bone marrow cell illustrating the margination of nuclear chromatin (NC) characteristic of cells infected with herpes virus i.e. cytomegalovirus. Other viral particles are present in the cytoplasm which are analogous to the LIA viruses (type II) described by Fonson et al. (1969). These type II LIA viruses have a translucent core and measure 70 nm in diameter and are indicated by arrows in the micrograph. Mag. 25,500 x.
Figure 19. Electron micrograph of a cytoplasmic vesicle containing two virus types. Type I measures 100 nm (I) and type II 70 nm in diameter (II). Both particles appear to be budding (B) through the vesicle membrane indicated by the doubling of the membrane at the location of the virions. Mag. 42,500 x.
Figure 20. Electron micrograph of an acute bone marrow cell. Type I and type II viral particles are present and particles can be seen budding (B) from the cell surface. Numerous type II (II) particles are associated with the lamellar membranes (LM) in these infected cells. Mag. 42,500 x.
Figure 21. Electron micrograph of an acute bone marrow cell which has been pre-inoculated with purified EIA virus. The virus concentration in this cell has increased and both type particles are present as indicated by arrows labeled for type I (I) and type II (II) particles respectively. Mag. 32,000 x.
5) That one agent causes a disease called EIA in one locality and the disease caused by the other agent is sufficiently similar to also be called EIA.

It is certain, at least, that viral particles are found in infected cells that are not observed in uninfected cells. Virus isolated from the medium in which infected cells are cultured produced EIA when inoculated into a normal pony. This incriminates at least one virus as the causative agent of EIA.

C. Virus Isolation and Purification

Isolation and purification of the virus from infected EIA serum was most successful using Scheme B. A dilution to $10^{-5}$ of this preparation still revealed 45-90 nm particles in EIA negatively-stained preparations. Particles were not seen in dilutions beyond $10^{-2}$ in Scheme A and only through $10^{-4}$ in Scheme C. Thus Scheme B was chosen as the method of choice for virus purification. The principal disadvantage of Scheme A was the dilution factor from the DUAL column chromatography step. Adequate means of concentration of the viral effluent were not available, making the ultimate virus preparation too dilute to be useful. Virus preparations from Schemes B and C gave approximately equal concentrations. Sucrose density gradients were preferable to CsCl for subsequent animal inoculations. Although excess CsCl was removed by extensive dialysis, even a trace of CsCl left in the preparation would cause a shock reaction in the animal.
Likewise, virus preparations from Scheme C after dialysis had less noticeable effects on tissue cultures when inoculated than did CsCl preparations. Increased cell growth was observed in sucrose preparations unless the virus was diluted at least $10^{-1}$ in M199 prior to inoculation of the BMC. This increased metabolism may reflect the increase in sucrose or glucose in the medium.

An inoculum of 3.1 ml dialyzed virus isolated by Scheme B from EIA serum was inoculated into a normal pony intravenously. The first febrile response was 10 days post-inoculation. The pony was designated acutely-infected and was sacrificed 8 days later.

Subsequent to detection of viral particles in ABM cultures, medium from these cells was used for isolation of the EIA virus by Scheme C. Five ml of the purified viral preparation was dialyzed at 4°C in 0.1 M PBS pH 7.0 for 24 hrs. A normal pony was inoculated intramuscularly with the virus isolate. The first febrile response was 18 days post-inoculation and designated EIA positive by gel-diffusion test.

It may be concluded that some agent can be isolated from serum of an acutely-infected horse and ABM culture fluid that will experimentally produce an acute case of EJA.

D. Physicochemical Characterization

Subsequent characterizations of the EIA virus were carried out on pooled EIA preparations. The density determined by equilibrium density gradient centrifugation at 100,000 x g
Figure 22. Determination of the optical density at 260 μm and the density of each fraction from isolation of virus from equilibrium density gradient centrifugation. The O.D. peak fractions 13 and 14 correspond to the density of 1.146 g/cm³ reported for the EIA virus.
in 15% (4 ml) CsCl gradient revealed the absorbing peak at 260/280 nm CO in fractions 13, 14 to have a density of 1.145 g/cm³. This is consistent with 1.146 g/cm³ reported by Nakajima et al. (1969) for EIA virus purified by Scheme A obtained from infected HLC.

Staining of bone marrow monolayers with PAS-methylene blue and fuclogen stain revealed DNA concentrated areas in the cytoplasm of infected cells and occasionally in the nucleoplasm. Cells stained with methylene blue had blue nuclei with occasional blue areas in the cytoplasm surrounded by pink cytoplasm. This procedure is designed to demonstrate cellular basophilia which it did in the infected EIA cultures from ABM but was not observed in KB1 or CEM cells.

Fuclogen-stained ABM revealed DNA areas in the cytoplasm of approximately 20% of cells as well as the nucleus of these cells. ABM and CEM cells did not contain these pink to red dots in the cytoplasm. This technique was not found sensitive enough an assay for EJA due to the low titer of virus present.

Modified methods of Frefielder, Swesty, and Marmur for DNA extraction were compared. The comparative efficiencies of these methods of extraction are represented in Table 4. DNA cannot be detected by DPA if the sample is treated with DNAase. The method of choice was Marmur's because the yield of DNA was greatest. Marmur's method was utilized subsequently for extraction of DNA for EM preparation by Kleinschmidt procedure. The DNA thus obtained is illustrated in Figure 23.
Table 4. Comparison of DNA extraction methods from virus isolated from horse serum.

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\(^1\) Used for EM preparation
Figure 23. Molecules of DNA extracted from purified EIA virus and prepared by Kleinschmidt technique. Mag. 30,000 x.
It was observed that this DNA was linear. In the light of replication sequence similar to HSV-II, the DNA was expected to be linear which is consistent with DNA found in other herpes-like viruses.

Buoyant density of isolated DNA from the purified EIA virions revealed the molecule to have a density of 1.714 g/cm³ determined as follows: (Mandel and Karmur, 1968)

\[
P^{25°} = 10.9601 \frac{25}{D} - 13.4974
\]

\[
P = 10.8601 (1.400) - 13.4974
\]

\[
P_0^{25} = 1.707
\]

\[
P = P_0 + 4.2 w^2 (r^2-r_0^2) \times 10^{-70} \text{ g/cm}^3
\]

\[
P = P_0 + 0.0092 (r^2-r_0^2) \text{ g/cm}^3
\]

\[
P = \text{density of EIA DNA}
\]

\[
P_0 = \text{density of standard E. coli DNA}
\]

\[
w = \text{speed of rotation in radians/sec}
\]

\[
r = \text{distance ex center of rotation (cm) for EIA DNA}
\]

\[
r_0 = \text{distance from center of rotation for E. coli DNA}
\]

\[
P = 1.707 + 0.0092 (1.5^2-1.2^2)
\]

\[
P = 1.714 \text{ g/cm}^3
\]

The GC content was determined by the method of Schildkraut et al. (1962) as follows:

\[
\operatorname{GC} = \frac{P_0 - 1.660}{0.098}
\]

\[
\operatorname{GC} = \frac{1.714 \text{ g/cm}^3}{0.098} = 55.18
\]
Figure 24. Densitometer tracings of ultraviolet photographs of CsCl buoyant density gradients of *E. coli* DNA and EIA virus extracted DNA.
Results of the Joyce Label densitometer tracings of the UV photographs of CsCl buoyant density gradients using a E. coli DNA standard are illustrated in Figure 24.

Isolation of DNA from EIA virus was not consistent with the work of Nakajima et al. (1970). However, Moore et al. (personal communication) reported that EIA is a DNA virus. Some data recently presented were interpreted as indicative of the presence of RNA (Kono, Yoshino, and Fukunaga, 1970b). These experiments with IUDR, however, could be viewed in a different light. That IUDR stops virus is reestablished by addition of thymine is not necessarily a restricted need of RNA virus replicative cycle. A DNA virus would also require DNA synthesis for replication of its own genome before further synthesis of virus production could continue in the viral replicative cycle. Thus it is felt that these data may be just as easily interpreted as an indication of a DNA virus as a RNA one.

Martin's method (1969) was used to extract RNA specifically from the EIA virus preparations. The extract, when tested by the orcinol test, did not reveal RNA to be present.

E. ImmunoIogical Characteristics

1. Immunodiffusion Studies

Immunodiffusion data for EIA virus and various antisemum or antibody preparations are represented in Table 5. Purified pooled EIA virus collected from fractions with
Table 5. Immunodiffusion test results between DIA purified virus as antigen and various antisera.

<table>
<thead>
<tr>
<th>Source of Antibody</th>
<th>Anti EIA Rabbit Serum</th>
<th>Normal Rabbit Serum</th>
<th>Anti EIA Rabbit Serum Adsorbed with DIA Virus</th>
<th>Calf Serum</th>
<th>H-21 Acute EIA Horse Serum</th>
<th>H-11 Acute EIA Horse Serum</th>
<th>H-71 Normal Horse Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified EIA virus</td>
<td>+1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Ether Treated purified DIA virus</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H-21 Whole Serum (Acute)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Normal Equine Serum pellet</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Virus isolated from acute bone marrow culture fluid</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Antigen</td>
<td>Anti EIA Rabbit Serum</td>
<td>Normal Rabbit Serum</td>
<td>Anti EIA Rabbit Serum Adsorbed with EIA Virus</td>
<td>Calf Serum</td>
<td>H-21 Acute EIA Horse Serum</td>
<td>H-11 Acute EIA Horse Serum</td>
<td>H-71 Normal Horse Serum</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>-----------------------</td>
<td>---------------------</td>
<td>---------------------------------------------</td>
<td>------------</td>
<td>----------------------------</td>
<td>----------------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>Pellet from normal bone marrow culture fluid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pellet from calf serum</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pellet from calf bone marrow culture fluid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pellet (100,000 x g) from K-21 serum</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Pellet from K-71 normal serum</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

1 These results represent 10 experiments - 9/10 to 10/10 = +
   2/10 to 3/10 = +/-
   0/10 to 2/10 = -
with density of 1.13-1.16 g/cm³ isolated from EIA infective serum was used as antigen. These viral particles were examined in the electron microscope by negative-staining and were found to be 45 and 80-100 nm in diameter and roughly spherical.

Precipitin lines were strongest and easiest read when undiluted horse serum and virus were used. When the virus preparation was diluted 2, 4, 6, 8, and 10 fold no precipitin arcs could be detected after a 2 fold dilution. Likewise, dilution of the horse serum containing EIA antibodies more than 2 fold resulted in loss of detectable precipitate arcs.

Nakajima et al. (1971) reported success with immunodiffusion using virus purified from HLC infected with EIA. The virus preparation had a density range of 1.12-1.19 g/cm³ and an OD₂₈₀ between 1.5 and 2.0. The sedimentation coefficients measured by analytical ultracentrifugation were between 110 and 120 S (Nakajima et al., 1971).

Using several strains of EIA virus in addition to the Wyoming strain, Nakajima et al. (1971) found all formed precipitin lines that connected with one another. This suggest that antibodies in the serum reacting with the antigen were all immunologically related. Serological reactivity was lost when positive horse serum was added to the antigen (viral preparation) (Nakajima et al., 1971). Results with immunodiffusion using purified virus are consistent with these findings (Table 5). Antigenic reactivity was lost when mixed
with horse serum containing LIA antibody (H-11 serum) prior to use in immunodiffusion experiments.

Complement-fixing and neutralizing antibodies have been demonstrated in LIA (Nakajima et al., 1971; Kobayashi et al., 1969; Kono and Kobayashi, 1966). However, the CF antibody is present for only a short period of time after the first febrile attack of the disease (Kono and Kobayashi, 1966). The neutralizing antibody on the other hand has been identified to persist for a longer period (Kono, 1969). The reaction is type specific. Nakajima et al. (1971) investigated sera from 9 horses experimentally infected with LIA at various stages of the disease to determine the time of precipitating antibody and its longevity. The antibody appeared 3-2 weeks after the first febrile attack and persisted at least 5 months after onset of disease. He considers this precipitating antibody specific for LIA but not against intact LIA virions. An internal component of the virion when released by uncoating would account for the antigenic properties described by Nakajima.

Coggins and Norcross (1970) recently reported an immunodiffusion diagnostic test for LIA using an infected horse spleen preparation as antigen. This test was very successful in demonstrating the presence of precipitating antibody. The relationship between the antibody active against the splenic extracted antigen and the purified virus antigen used by Nakajima et al. (1971) is not known.
Some information is available to explain the difference in antigen found in the splenic preparations in contrast to purified viral preparations. Norcross (personal communication), Coggins (personal communication), and Benson (personal communication) reported this reacting antigen to be a protein with a molecular weight of 27,500 daltons, an S value of 2.1 and a density of 1.18 and 1.19 g/cm³.

2. Fluorescent Antibody Studies

The fluorescent antibody (FA) technique has been widely used in studies with oncogenic viruses, and its application in detecting viral antigen in different preparations has been well-documented (Brown et al., 1966; Malagren et al., 1960; Rollers and Munroe, 1960; Payne et al., 1966). Kotturidis and Luginbuhl (1967) used the FA technique to examine cell cultures and bone marrow smears for detection of the Marek's disease (MD) agent. The pathology of MD is suggestive of a leukemia process although a herpes virus has been shown to be involved (Menle, personal communication). It has been suggested that the replication of EIA virus is similar to that observed for leukemia viruses. A process similar to that described for MD may be operative. FA techniques were employed as a means of detecting the EIA agent in the bone marrow cultures.

Immunofluorescence was not observed in CBM or NBM cells, thus eliminating possible non-specific fluorescence. When CBM cells were preadsorbed by inoculating with isolated EIA virus 1 to 3 days prior to FA studies, 10 to 20% of the
cells had cytoplasmic fluorescence. Preadsorbing of ABM cells revealed 20 to 30% of the cells contained EIA antigen. All fluorescence was removed by adding EIA antiserum to the virus isolate prior to inoculation of the BM cultures.

The immunofluorescence of ABM cells was cytoplasmic in 20 to 30% of the cells through the seventh passage. When isolated EIA virus was preadsorbed onto ABM cells, 30 to 40% of the cells had cytoplasmic fluorescence. Most of the ABM cells had cytoplasmic and nuclear fluorescence in BMC cells fixed 3 days after inoculation of EIA virus isolate. Several cells would be observed in close association and fluoresce as one distinguishable mass.

Antiserum was prepared in one rabbit against purified EIA virus. The antiserum was shown to be specific for the virus by gel diffusion test (Table 6). A precipitin arc was not formed with control serum from a rabbit inoculated with normal horse serum.

EIA virus adsorbed with antiserum blocked fluorescence and no fluorescence was seen in cultures with normal horse antiserum or in NBM or CRM uninfected cells. The specificity with which this fluorescence was observed in infected BMC indicated that the antiserum was produced against the agent responsible for EIA. It is further supported by the fact that when this viral preparation was inoculated into a normal horse the animal subsequently died of EIA.

It has recently been reported (Ushimi et al., 1971) that EIA may be also detected by use of indirect
Table 6. Indirect fluorescent antibody detection of EIA viral antigen in cell culture.

<table>
<thead>
<tr>
<th>Cell Culture</th>
<th>Number of Specimens</th>
<th>Special Treatment of Cell Cultures</th>
<th>Degree of Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf Bone Marrow</td>
<td>14</td>
<td>Adsorbed with EIA virus prior to fixation</td>
<td>+ to ++</td>
</tr>
<tr>
<td>Calf Bone Marrow</td>
<td>12</td>
<td>Unadsorbed</td>
<td>0</td>
</tr>
<tr>
<td>Calf Bone Marrow</td>
<td>8</td>
<td>Controls</td>
<td>0</td>
</tr>
<tr>
<td>Normal (Horse) Bone Marrow</td>
<td>25</td>
<td>Adsorbed with EIA virus prior to fixation</td>
<td>++ to +++</td>
</tr>
<tr>
<td>Normal (Horse) Bone Marrow</td>
<td>23</td>
<td>Unadsorbed</td>
<td>0 to +</td>
</tr>
<tr>
<td>Normal (Horse) Bone Marrow</td>
<td>8</td>
<td>Controls</td>
<td>0</td>
</tr>
<tr>
<td>Normal (Horse) Bone Marrow</td>
<td>19</td>
<td>Fed with H-21 (EIA) serum prior to fixation</td>
<td>0 to +++</td>
</tr>
<tr>
<td>Acute (Horse) Bone Marrow</td>
<td>11</td>
<td>Adsorbed with EIA virus prior to fixation</td>
<td>+++ to ++++</td>
</tr>
<tr>
<td>Acute (Horse) Bone Marrow</td>
<td>24</td>
<td>Unadsorbed</td>
<td>++ to +++</td>
</tr>
<tr>
<td>Acute (Horse) Bone Marrow</td>
<td>2</td>
<td>Unadsorbed</td>
<td>0</td>
</tr>
<tr>
<td>Acute (Horse) Bone Marrow</td>
<td>8</td>
<td>Controls</td>
<td>0</td>
</tr>
</tbody>
</table>
Immunofluorescence technique. In this work Ushimi used HLC infected with EIA virus. EIA viral antigen was detected in the coverslip preparations 2, 3, and 4 days after EIA viral inoculation of the HLC. There was a progressive development of viral antigen with the lapse of time following inoculation. The staining was almost entirely confined to the cytoplasm of infected cells and occasionally the cell surface fluoresced brightly. Moreover, Ushimi (1971) reported that the virus nucleic acid was RNA although the techniques used were not given. This is in contrast to the DNA extracted from purified virus as reported in this present study. It is, therefore, suggested that two viruses may be present in HLC. Since the viral preparation used by Nakajima was from HLC-infected medium, two viruses may be present. One possible isolate is equine arteritis virus which is an RNA virus with a density of 1.20 g/cm$^3$ and is 60 nm in diameter with a nucleoid 35 nm in diameter (Naess and Bohn, 1970). If both are present then antiserum to the viral preparation would contain antibodies to both and be conjugated and thus show fluorescence in IFA tests for both viruses. If the RNA viral concentration is greater than the DNA virus population it could produce these results. The virus used to produce antiserum for IFA by Ushimi was not reported to cause EIA by subinoculation (Ushimi, 1971).

Attempts to use direct fluorescent antibody techniques for detection of EIA antigen in HLC were unsuccessful (Ushimi, 1971). Fluorescence was very weak and no difference was detected between EIA cultures and the controls.
Table 7. Direct fluorescent antibody detection of equine herpes virus in cell cultures

<table>
<thead>
<tr>
<th>Cell Culture</th>
<th>Number of Specimens</th>
<th>Special Treatment of Cell Cultures</th>
<th>Degree of Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf Bone Marrow</td>
<td>4</td>
<td>Adsorbed with EIA virus prior to fixation</td>
<td>+</td>
</tr>
<tr>
<td>Calf Bone Marrow</td>
<td>10</td>
<td>Unadsorbed</td>
<td>0</td>
</tr>
<tr>
<td>Normal (Horse) Bone Marrow</td>
<td>4</td>
<td>Adsorbed with EIA virus prior to fixation</td>
<td>+</td>
</tr>
<tr>
<td>Normal (Horse) Bone Marrow</td>
<td>10</td>
<td>Unadsorbed</td>
<td>0 to +</td>
</tr>
<tr>
<td>Acute (Horse) Bone Marrow</td>
<td>4</td>
<td>Adsorbed with EIA virus prior to fixation</td>
<td>+</td>
</tr>
<tr>
<td>Acute (Horse) Bone Marrow</td>
<td>7</td>
<td>Unadsorbed</td>
<td>+ to +++</td>
</tr>
<tr>
<td>Acute (Horse) Bone Marrow</td>
<td>2</td>
<td>Controls</td>
<td>0</td>
</tr>
</tbody>
</table>
Direct IF studies were conducted to ascertain if EIA and EHV had common antigenicity or if BM cells harbored EHV. Conjugated FITC anti-EHV serum was obtained from Dr. Kemény at NADL and used in DFA tests with identical cell cultures used in IFA work. No fluorescence was obtained as shown in Table 6 unless the cells were preinoculated with EIA purified preparation. It was later learned that this particular conjugate was prepared in a chronic EIA horse. Therefore, it is felt that the fluorescence thus obtained after infection with EIA is due to anti-EIA antibodies present in the FITC anti-herpes virus (EIA horse serum). The fluorescence obtained is detection of EIA and not EHV. If there were some EHV present fluorescence should have been obtained prior to absorption with EIA preparation.

The IFA test was a successful way to demonstrate the presence of EIA antigen in infected EMC. This procedure was more desirable than EM studies because of the low percentage of infected cells. Further, the data suggest that the EIA virus maintains a latent type of infection. This point is further substantiated by the production of EIA with virus isolated from the media from infected EMC. Therefore, these data suggest that the EIA virus resembles the equine CMV group of herpes viruses.
SUMMARY AND CONCLUSIONS

Investigations were conducted to isolate, purify, and characterize the EIA virus. Virus was isolated by sucrose and CsCl equilibrium density-gradient centrifugation from infected serum and tissue culture fluid from EMC.

Electron microscopic examination of the purified preparations revealed 45-50 nm and 70-120 nm diameter particles. The virions were spherical to pleomorphic. One band from ultracentrifugation in CsCl density gradient had a density of 1.146 g/cm$^3$. Inoculation of this fraction into a normal horse produced an acute infection of EIA.

Likewise, the same-sized virions were isolated from the pooled medium from infected BMC. Inoculation of this viral preparation into a normal pony also produced EIA and was positive by the immunodiffusion test.

Nucleic acid from the purified virions was found to be DNA. EM observation by the Kleinschmidt technique showed the DNA to be a linear double-stranded molecule. Buoyant density studies revealed the DNA to have an approximate density of 1.714 g/cm$^3$.

EMC allowed viral propagation as well as the HLC used previously. Thin-sections of infected BMC demonstrated two morphologically distinguishable viruses present. Viral-like
particles were not seen in similar preparations of NRK cells. An increase in the number of viral particles detected was observed when BMC was adsorbed with purified virus. No CPE was demonstrable in vitro, however.

One of the two virus types resembles BHV. Type I has a diameter of 80-120 nm, and electron dense nucleoid and outer membrane. The nucleoids of this virus measure 45-50 nm in diameter.

Type II virus was 70 nm in diameter with an electron translucent core and electron dense outer coat. Both type particles are assumed to be present in the purified virus preparation and of approximately the same molecular weights since only one band is formed in an equilibrium density gradient. Due to the low percentage of infected cells, the EM thin-section technique was not a good method for detection of EIA in the cultures.

Immunological techniques were more satisfactory in detection of the virus in BMC. The IFA method was a successful way to demonstrate the EIA viral antigen present in BMC. Lack of nonspecific fluorescence and production of specific EIA antiserum lend confidence to these data. Fluorescence was observed in the cytoplasm. Occasionally the nuclear membrane was bright in early infected cells and the cell periphery fluoresced in late infection. The percent of cells which were harboring viruses was low as indicated by EM studies.

Antibodies to the EIA virus could be demonstrated in horse serum by immunodiffusion. The intact EIA virus as well
as inactivated ether-treated virions were reactive antigens in the immunodiffusion tests.

Characteristics observed in EM thin-sections for these viral particles resemble the CMV group of herpes viruses. These characteristics were: margination of chromatin, cytoplasmic vesicles containing virus, budding from cytoplasmic vesicles and cell surface, presence of envelopes, DNA content and low percent of infected cells. To conclude that LIA virus is associated with or belongs to the CMV group requires further research at the molecular level.


VITA

Virginia Ann Lewis Boyd, daughter of Mr. and Mrs. Fletcher W. Lewis was born November 15, 1944 in Shreveport, Louisiana. She was graduated from Fair Park High School in 1962. In June, 1964 she married James F. Boyd of Baxaliloches, Louisiana. In August, 1965 she received her B.S. degree in Chemistry from Northwestern State College. She was employed as a laboratory technician for Martin Army Hospital until August 1966. In January, 1968 she received her M.S. degree in Microbiology from Northwestern State College of Louisiana. Until June 1969 she taught Microbiology, Genetics, and General Biology at Jacksonville State University of Alabama. She had two children, Kathryn Ann born January 4, 1965 and David Gregory born December 10, 1965. In June 1969 she entered Louisiana State University and was appointed on the National Institutes of Health Training Grant, and is a candidate for the Ph.D. degree in Microbiology, with a minor in Biochemistry in August 1971.
Candidate: Virginia Ann Lewis Boyd

Major Field: Microbiology

Title of Thesis: Isolation and Characterization of Virus from Horses Infected with Equine Infectious Anemia

Approved:

[Signature]

Major Professor and Chairman

[Signature]

Dean of the Graduate School

EXAMINING COMMITTEE:

[Signature]

[Signature]

[Signature]

Robert J. Anderson

Date of Examination:

July 14, 1971