Equine Infectious Anemia Virus: (1) the Generation of Virus Variant Candidates. (2) Attempts to Titrate the Virus by Three Different Methods.

Alberto Orrego uribe

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EQUINE INFECTIOUS ANEMIA VIRUS: (1) THE GENERATION OF VIRUS VARIANT CANDIDATES. (2) ATTEMPTS TO TITRATE THE VIRUS BY THREE DIFFERENT METHODS

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EQUINE INFECTIOUS ANEMIA VIRUS:
(1) THE GENERATION OF VIRUS VARIANT CANDIDATES
(2) ATTEMPTS TO TITRATE THE VIRUS BY THREE DIFFERENT METHODS

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 Veterinary Microbiology and Parasitology

by
Alberto Orrego Uribe
D.V.M., Universidad de Caldas, 1966
M.P.V.M., University of California, 1974
August 1983
DEDICATION

To my wife and daughters for their endurance and understanding.
To my family.
To my family-in-law, especially to Fabio and Teresa who rested in the peace of our Lord during the time this work was conducted.
ACKNOWLEDGEMENTS

Appreciation is first expressed to Dr. C.J. Issel, who served as the author's Major Professor and adviser, for his suggestions and encouragement during the experimental work and preparation of this dissertation.

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### CHAPTER I

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### CHAPTER II

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ABSTRACT

Six serial passages of a cell-adapted strain of EIAV were conducted in Shetland ponies. The 16 recipient ponies became agar-gel immunodiffusion test positive by 25 days after inoculation, and it was observed that the virulence of this cell-adapted strain increased through the successive passages. Clinical signs were closely monitored and recorded, and macroscopic as well as microscopic lesions of EIA were also recorded and evaluated. The effect of EIAV upon blood components was determined after several of the febrile episodes, and the effect of the immunosuppressant dexamethasone on infected ponies was also studied. EIAV was isolated in fetal equine kidney cells cultures from 90% of plasma samples collected during febrile episodes and from 51% of plasmas collected at afebrile periods.

The cross serum-neutralization test was carried out for EIAV isolates and sera collected from two experimental ponies. It was found that serum collected shortly after EIAV isolation did not neutralize the virus isolate, but the serum neutralizing activity increased in samples collected thereafter. None of the sera showed neutralizing activity against EIAV isolated after their collection. These results suggest an antigenic change in EIAV occurring in the pony which would allow for the virus persistence in its host.

Finally, a study was also carried out to determine whether the mixed culture cytopathogenicity assay, the syncytia infectivity assay, and the reverse plaque assay would be suitable to quantitate EIAV. These assays have been successfully used to titrate several murine leukemia viruses, bovine leukemia virus, and several strains of hog
cholera virus respectively. None of these assays, however, was found to be accurate and reliable to titrate EIAV.
GENERAL INTRODUCTION

Equine infectious anemia is one of the most important diseases of horses as it is present in almost any country in which horses are raised, causing heavy losses to the equine industry. Equine infectious anemia is one of the oldest viral diseases known to affect members of the Family Equidae.

The chronicity of the disease and the appearance of febrile relapses after the first febrile episode, have been the hallmark of equine infectious anemia, but also a puzzle for researchers who have attempted to explain why the horse remains an equine infectious anemia virus carrier forever, despite a normal immune response, and why the febrile relapses occur. It has been suggested that the periodic attacks of equine infectious anemia occur in concert with the generation and spread of novel strains of the virus, with the property to circumvent the host immune response and, consequently, to maintain a persistent infection in its unique host. So far, the nature of that antigenic variation in equine infectious anemia virus remains unknown.

It is the purpose of this research to generate sequential equine infectious anemia virus isolates for further serological and biochemical characterization that would lead in the near future to disclosure of the nature of the antigenic variation of equine infectious anemia virus and, therefore, to the preparation of immunoprophylactic or immunochemical protocols for the prevention and control of this elusive disease of horses.
CHAPTER I
Equine Infectious Anemia:
The Generation of Virus Variant Candidates
INTRODUCTION

Equine infectious anemia (EIA) is one of the most important diseases of horses as it affects a large number of members of the Family Equidae throughout the world, causing deaths, abortions, and low performance of chronically infected individuals. Similarly, the recurrent character of this disease has been a real challenge for researchers. The way the etiological agent is believed to escape the host immune response presents a real obstacle for the development of an effective immunogen against the disease; however, the fact that equine infectious anemia virus (EIAV) does not infect any other animal species is an important factor in its eventual future control. Finally, as EIA is a widespread disease, it has been known by several synonyms such as swamp fever, equine malarial fever, pernicious anemia, American surra, slow fever, mountain fever, and typhoid fever of horses.

The name "swamp fever" was given in the past to stress the belief that the disease was more prevalent in animals pastured in low-lying areas, but actually, the disease is present in horses living at high altitudes (9000 feet above sea level). EIA has been known to exist in the United States for about 80 years and statistics compiled by the United States Department of Agriculture show that the highest percentage of samples testing positive in the United States originated in Louisiana. It was found that from January 1971 to December 1974, the prevalence rates ranged from 24.1% in 1971 (n = 3,465) to 12.7% in 1974 (n = 8,996) and that EIA was more prevalent in the southwestern parishes (33.7% of 1,511 samples were positive) and that the lowest rate was observed in samples originating from horses in Orleans parish (3.8% of
1,147 samples were positive). In 1975, a survey was conducted in East Baton Rouge Parish to determine the prevalence of EIA by the agar gel immunodiffusion (AGID) test finding that 6.7% of the horses were infected. In addition, according to statistics compiled by the U.S. Department of Agriculture for fiscal years 1972 through 1975, antibodies to EIAV were 4 to 6 times more prevalent in samples from horses in the gulf coast states of Texas, Louisiana, Mississippi, Alabama, and Florida than elsewhere in the United States.

The incidence of EIA was determined by Orrego et al. in a band of horses experiencing an outbreak of the disease in 1976, where 11 horses had died due to EIA and 44.4% (36 out of 81) of the horses were positive reactors in the AGID test. A second test was carried out six months after the first examination and nine new positive horses were found, which meant that the incidence of the disease was 36% in that group of horses for the specified period of time. The remaining negative horses were tested by the AGID test every two months thereafter, finding that the incidence of the disease was 27% from April to June 1977 and 7.7% from July to September 1977. No new cases were detected between October 1977 and June 1978, probably due to the elimination of the positive reactors. The same authors, at the same time, carried out a survey in horses from the rural and urban areas near the investigated outbreak and found that both the prevalence and the incidence of EIA was much lower in these populations than in bands of horses raised under conditions of high density.
THE ETIOLOGY OF EQUINE INFECTIOUS ANEMIA

The etiological agent of EIA is a virus from the Family Retroviridae, Subfamily Lentivirinae (from the Latin, Lenti = slow). Hence, the virus is an RNA virus related to tumor viruses (Oncovirinae and Spumavirinae), yet, in the properties of the virus particle, EIAV resembles visna, maedi, and progressive pneumonia viruses more than other viruses from the family.

CHARACTERISTICS OF EIAV

Equine infectious anemia virus is spherical, enveloped, and the virion has a diameter of 80 to 100 nm. The icosahedral capsid contains a heavy molecular weight RNA genome (60 to 70 S) composed of subunits. The virus density is between 1.16 and 1.18 g/cm$^3$ in sucrose gradients. EIAV, like prototype retroviruses, matures by budding from external and internal cytoplasmic membranes, displaying a complex morphology characteristic of type C viruses, and contains a reverse transcriptase enzyme.

According to Matthews, viruses from the family Retroviridae contain about 60% protein, 35% lipid, and 3.5% carbohydrate by weight. The polypeptide composition of EIAV was determined by Parekh et al., by using purified radioactive leucine or glucosamine-labeled EIAV of the cell-adapted Wyoming strain by two procedures: guanidine hydrochloride gel filtration (GHCL-GF) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). By the first procedure, the chromatographic analysis of the radioactive EIAV revealed six distinct peaks of radioactivity, as follows: the first protein(s) to elute from GHCL-GF were contained in the void volume fractions of the column indicating an apparent molecular weight of at least 100,000. The next EIAV protein
eluted as a relatively broad peak with an apparent molecular weight of about 74,000. This component was followed by the major EIAV structural polypeptide (p26) which displays a molecular weight of 26,000, and in succession, by three additional components of 15,000 (p15), 11,000 (p11), and 9,000 (p9) molecular weight, respectively. By the SDS-PAGE, four major low molecular weight polypeptides (p26, p15, p11 and p9) were revealed and in addition, other heavy molecular weight components were resolved, which included two polypeptides of 90,000 (gp90) and 45,000 (gp45) molecular weight which were glycosylated, and four minor non-glycosylated proteins designated as p70, p61, p30, and p23. From the same study, the authors also concluded that EIAV and Friend murine leukemia virus (FLV) contain remarkably similar structural components, but they do slightly differ in the apparent molecular weight of their polypeptides.

**CLINICAL DISEASE**

Horses infected with EIAV have a variable clinical disease course. The prominent signs include pyrexia, depression, weight loss, and anemia. The incubation period in most experimentally infected horses is less than 30 days, but it is dose dependent and may extend to 90 days in horses receiving small quantities of virus by the subcutaneous route. Kemeny et al. reported that the incubation period following subcutaneous inoculation of infective serum varied inversely with the dose increasing from 6 days with 100 ml to 23 to 30 days with 1.0 ml of a 10^-6 dilution of the same serum.

The most characteristic clinical sign is pyrexia which usually lasts 3 or 4 days, although it may last several weeks in some animals.
During any episode of clinical disease, the animal may die. Most horses survive the first febrile episode and subsequently experience multiple exacerbations of clinical disease, which are interspaced by several days or weeks of clinical normality. This type of clinical course is the most common in experimentally infected horses. After weeks or months of recurring clinical disease, the animals may become quiescent and not show signs for months or years. Animals that have been apparently normal for a year or more may suddenly reexperience an acute febrile episode. All horses have persistent viremia regardless of this clinical course.\(^1\,2\,0\,2\,2\) Thus, the spectrum of disease extends from acute disease, resulting in death, to asymptomatic carriers.\(^1\,4\)

Once a horse is infected with EIAV, it is thought to remain a carrier of the virus for life.\(^2\,0\) Several investigators have tested blood from infected horses systematically over a period of 12 to 18 years and have consistently found the blood to be infectious for susceptible horses.\(^2\,3\,–\,2\,5\) Early workers assumed that the persistence of EIAV in the blood stream of an infected horse resulted from a lack of an immunological response by the host to the invading agent; however, it has been proven that concept was misleading and that, in fact, the body produces an abundance of certain types of specific antibody as a result of exposure to EIAV.\(^2\,0\)

Acute EIA is most often associated with the first exposure to the virus, with fever and hemorrhages evident from 7 to 30 days after infection. Acute disease is thought to be associated with massive virus replication in and destruction of infected macrophages. Horses in the initial phase of acute EIA will be seronegative because the immune
system has had insufficient time to respond to the viral antigen. During the peak of the febrile response with acute EIA, viremia of $10^6$ horse infectious doses/ml of whole blood is often found and EIAV antigen may be extracted from splenic tissue at that time. The initial acute phase of EIA infection may not be seen by the veterinarian unless there is an epizootic of the infection in a group of horses. Even then, the horses must be under close supervision before the initial fever and anorexia are detected. Neither anemia nor edema are seen at this stage.

The more classical clinical signs of chronic EIA are loss of weight, anemia, and edema which are seen later during recurring cycles of the illness and which appear at biweekly intervals. The frequency and severity of clinical episodes in horses with chronic EIA usually decline with time, about 90% of them occurring within one year of infection. The earliest deaths due to EIA usually do not occur until 4 weeks after infection. Horses with subacute and chronic EIA will be seropositive. The EIAV titer in horses with chronic EIA varies but it is highest during the periodic febrile episodes. Chronically infected horses have the most marked microscopic lesions. The periodic flare-ups of chronic EIA may be due to the production and release of novel antigenic strains of virus, which are not neutralized by existing antibodies. The subtle changes in the phenotype of EIAV have been referred to as "antigenic drift" and may occur because of minor mutations in EIAV-RNA.

Many of the clinical signs in horses with EIA may be referable to changes induced by immune reactions to viral products. An example of
this may be the anemia associated with EIAV infection. The pathogenesis of the anemia has not been elucidated, but since EIAV possesses a hemagglutinin, virus may attach to erythrocytes and specific antiviral immunoglobulins then attach to viral proteins. The immune complexes on the cell surface would then attract complement. Complement coated erythrocytes are found in anemic EIAV-infected horses and the affected erythrocytes are then phagocytized by leukocytes. Finally there is an important form of EIA in a high percentage of EIAV infected horses which are detected by the AGID test, i.e., horses that do not show clinical illness associated with the infection, but do have a persistent infection.

The hallmark of chronic EIAV infection is the periodic appearance of clinical episodes of the disease which, according to Kono, are due to the formation of novel strains of EIAV that make possible the persistence of the virus in its host, despite a normal host immune response. These changes in the antigenicity of the virus are known as "antigenic drift" which is considered in the following.

The Case of Antigenic Drift - In various viral diseases like visna of Ovidae and EIA of Equidae, it has been found that, despite a normal host immune response to the viral infection, the individual remains infected for years. One mechanism, known as antigenic drift, by which these viruses produce antigenic variants allows the virus to temporarily escape from previously formed neutralizing antibody and from the cell mediated immune response. Changes in antigenicity are better known in other viruses as influenza virus, in which the B and C strains are
believed to undergo less antigenic variation than the A strain. It seems that the variability of human influenza viruses involve several mechanisms that lead to the emergence of different subtypes (by reassortment of surface protein genes between different strains, possibly involving animal strains), to variation within subtypes by point mutations occurring in genes coding for surface, as well as for nonsurface proteins, or by deletions and insertions in genes coding for hemagglutinins and neuraminidases. Yet, the reassortment of genes (genetic recombinations) induces major changes in the virus antigenicity which are recognized as "antigenic shift." These antigenic shifts occur at long intervals of time (several years) and may involve recombination and genetic interchange between a human and an animal influenza virus. Some other viruses, like foot and mouth disease virus, vesicular stomatitis virus, rabies virus, and polio virus, undergo antigenic variations attributed to either persistence or high input short-term infections which can provide conditions in which the inherent high mutation rates of RNA viruses are reflected in high rates of genome changes. Antigenic drift, on the other hand, is believed to occur due to accumulation of point mutations, which are cumulative, and sequential changes in the nucleotides that lead to changes in the amino acid sequences and therefore to antigenic variation.

In addition to antigenic variation in several viruses, antigenic variation in human and animal trypanosomes has been also well studied; as a matter of fact, humans and domestic animals infected with pathogenic African trypanosomes normally do not survive infection even though immune responses are mounted against parasite antigens.
Furthermore, in the case of trypanosomes, the mechanism for the formation of new antigenic variants of the parasite corresponds to the expression of biochemically and serologically distinct variant specific surface antigens (VSSA) which arise by direct mutation under immune pressure, but the intimate mechanism that triggers the antigenic variation is left unexplained. Nevertheless, it might be that virus variants arise due to RNA genome mutations and a population equilibrium is established. When that equilibrium is disrupted because of cell mediated immune pressure (infected cells are recognized and destroyed), some of the variants would be destroyed but some, or at least one, would escape the host immune response and would multiply, becoming the predominant strain. Antibody would be made against the novel strain of the virus and the horse would remain apparently normal for an unpredictable period of time before suffering a new febrile episode due to the generation and replication of a new EIAV variant.

Kono et al. with the aim of studying the antigenic variation of EIAV, inoculated 5 horses (subcutaneously) with a suspension containing \(10^4\) TCID\(_{50}\) of a "cloned" virus known as V70; as a result, all the recipient horses became AGID test positive by 24 post-inoculation day (PID), experienced the first febrile attack between 18 and 40 PID, and had an average of 3 febrile relapses during 155 days of observation. In addition, the infectivity of sera collected at the time of febrile relapses was found to fluctuate between \(10^{1.0}\) and \(10^{4.5}\) TCID\(_{50}\) of EIAV/0.5 ml. The neutralization activity of the sera collected from the same horse, at each one of the febrile episodes was tested against the virus isolated from the same material and against the parental virus
(inoculum, V70). The results showed that sera collected before inoculation and on 20 PID did not have neutralizing activity against any of the virus isolates (virus was isolated on PID 20, 44, 62, 83, and 155), while some neutralizing activity against the parental virus and the first virus isolate was detected in serum collected at 40 PID. On the contrary, sera collected at 62, 83, and 155 PID, failed to neutralize their correspondent virus isolate, but did neutralize all the virus isolates formed before serum collection. Equally important, the tested sera did not neutralize any virus isolated after their collection.

In another phase of Kono's work, he tried to demonstrate the immunological specificity of 5 viruses sequentially isolated from pony number 493, and 5 horses were inoculated with $10^6$ TCID$_{50}$ of the "cloned" isolates EIAV subcutaneously. The sequential virus isolates were reacted with sera collected from the same pony at different times after inoculation, and as a result it was reported that the neutralizing effect of the sera was not restricted to the respective homologus isolates only, but overlapped to viruses preceding or following them in the sequence of isolates. This result is not consistent with the neutralization data from Kono's inoculations with "V70" and suggests that some antigenic determinants are common between the EIAV variants.

Finally, the antigenic stability of one of the virus isolates was tested at different times in horse leukocyte culture by using homologus and heterologus antisera and, as a result, each passaged virus
showed the same and unaltered serological reactivity.21

Gutekunst et al.16 carried out a work with EIAV by using the equine dermal cell-adapted strain of the virus, which was adapted by Malmquist45 with the purpose of determining whether the virulence of this strain of EIAV would be modified by 200 passages in tissue culture when inoculated into ponies, would induce immunity against the disease, would revert to virulence in vivo, or would undergo antigenic alteration in the host. It was found that the onset of febrile response came later in ponies inoculated with high passaged EIAV than in ponies inoculated with low passaged EIAV (8 PID for EIAV passaged 2 times, and 121 PID for EIAV passaged 200 times); in addition, the authors found a correlation between number of passages and kinetics of positive AGID test samples.

Three of the four ponies inoculated with EIAV passaged 200 times had two to five intermittent febrile responses, beginning 70 to 126 PID. Each febrile response lasted 3 to 6 days and peak temperatures were between 38.6 and 41.5°C. One of the ponies had no increase in temperature before 202 PID. All became AGID test positive from 36 to 49 PID. Equine infectious anemia virus was isolated from primary pony leukocyte cultures at varied intervals from three of four ponies having intermittent febrile responses, yet, from one of the ponies the virus was isolated only once.

The cross-neutralization test carried out with the virus isolated, yielded expected results, i.e., neutralization did not occur with serum collected before EIAV inoculation, the sera collected at the time of virus isolation showed a low level of neutralization, which increased in subsequently collected serum samples. Finally, the virus isolates did
grow in pony leukocyte culture, known to support the propagation of wild types of EIAV.

**TRANSMISSION OF EQUINE INFECTIOUS ANEMIA**

The most important means of natural transmission of EIAV is the transfer of blood from the infected horse by blood feeding insects, especially horse flies and deer flies. The virus will be transmitted from one horse to another only when the feeding of the vector has been interrupted and the vector has to complete its meal in a different horse. So, even though both virus and horse flies are abundant, if the horse flies complete their blood meal on one horse there will be no transmission.

The mechanical transmission of EIAV by *Tabanus fuscicostatus* was investigated and as a result, in 1 of 7 transmission trials, a single horse fly transmitted EIAV from an acutely infected pony to a susceptible pony. It was also found that groups of horse flies isolated for 3, 10, or 30 minutes before refeeding transmitted EIAV, whereas those isolated for 4 or 24 hours did not. In another work by Hawkins et al., all the attempts to mechanically transmit EIAV by *T. fuscicostatus* to Shetland ponies from acutely infected donors were successful, even with a low number of fly bites. All mechanical transmission attempts from chronically infected ponies and biological transmission attempts from acutely infected ponies, however, were unsuccessful. Interestingly, transmission of EIAV by *T. sulcifrons* and *Stomoxys calcitrans* had been reported by earlier workers. There seems to be sufficient evidence to indicate that the disease may spread from one animal to another by simple contact, without the intervention of
insects (from contaminated food or water) or from infected animal
secretions. However, Kono et al. were unable to isolate EIAV from
urine and feces of 4 horses acutely infected with the virus. Equine
infectious anemia virus is actually transmitted to susceptible horses by
careless use of hypodermic needles, syringes, tattooing instruments, and
other piercing tools. Williams et al. detected EIAV on hypodermic
needles held at 25°C for 96 hours, but the virus was not detected 120
hours after the needles were dipped in solution of EIAV (10^8 TCID_{50}/ml).
On the other hand, in utero transmission of EIAV also occurs according
to Coggins and Kemen et al. who found that 25% of the foals from
infected mares get infected in utero and usually die shortly after
birth.

DIAGNOSIS OF EQUINE INFECTIOUS ANEMIA

Equine infectious anemia can be only suspected on the basis of a
clinical history. Hence, laboratory procedures are used to detect EIAV
from a variety of sources, and EIAV antibody from the serum of test
horses. The complement fixation test (CFT) was one of the first tests
to be used to detect EIAV antibody, but it has not been satisfactory
for the diagnosis of EIA because IgG(T) in the horse does not readily
fix complement (IgG fixes equine complement by the classic pathway
originating at Cl, but IgG(T) does not); yet, IgG(T) does not interfere
with the AGID test. The serum neutralization test has been used
as a research tool, but has limited usefulness in the diagnosis
of EIA. The same can be said of the hemagglutination-inhibition
test, while serologic tests such as fluorescent antibody, radioimmunoassay,
and enzyme immunoassay have been utilized to detect
EIAV or EIAV antibody. These tests are more sensitive than the AGID
test (as they detect minute amounts of antigen or antibody), but appear to be less specific. Thus, they might be an aid for detection when antigen or antibody concentrations are low, which would yield a negative AGID test.8

The AGID test has been the most useful test for the diagnosis of EIA59,60 as it accurately and reliably detects EIAV antibody. In fact, a correlation between precipitating antibody and EIA viremia has been demonstrated.59 Nakajima et al.61 studied the suitability of AGID test antigen for the diagnosis of EIA based on the following assessable considerations: (i) the antigen must be identical in its reactivity with an antigen prepared from highly purified EIAV; (ii) the antigen reacts identically against serum samples from horses infected with any strain of EIAV; (iii) the antigen forms only one distinct precipitation line against reference positive serum; (iv) the antigen reacts with any serum samples which contain precipitating antibodies ranging from high and low levels of antibody titer; and (v) the antigen does not show any specific reaction against serum samples from noninfected horses. On the other hand, many studies have been conducted and reported concerning the protocols for the AGID test59-66, and the test has been a valuable tool for the diagnosis of EIA as well as to determine the presence of the infection in many countries throughout the world.3,4,5,61,62,67 However, the test has some limitations: (i) no acute or early stages of the disease are detected; (ii) foals nursing from infected dams may give a false positive result; (iii) chronically infected horses may yield a negative test, and (iv) adequate experience is required to interpret the test results.60,62
The diagnosis of EIA can be assisted by clinical pathological procedures and by pathological alterations. In fact, clinical pathological alterations are related to the activity of the disease in a horse\textsuperscript{1}, while asymptomatic carriers have few detectable changes. The packed-cell volume and red blood cell counts decline during active disease.\textsuperscript{1,8,14,20} Siderocytes are found in variable numbers and the white blood cell counts vary, but associated with a decrease is a relative lymphocytosis as a result of a decrease in granulocytic type cells.\textsuperscript{1}

The most marked gross pathologic lesions are splenomegaly, lymphadenopathy, accentuated hepatic lobular structure, anemia, widespread hemorrhages, edema, and emaciation.\textsuperscript{26} Microscopically, there is marked lymphoid and reticuloendothelial cell proliferation, and infiltration occurs in the periportal and perisinusoidal areas of the liver. The Kupffer cells increase in number, become enlarged, and many contain hemosiderin. Proliferative glomerulitis characterized by irregular granular deposits of globulin and complement along the basement membrane and in the mesangial areas occurs in infected horses.\textsuperscript{14}

**EFFECT OF ADMINISTRATION OF IMMUNOSUPPRESSIVE DRUGS**

As the mechanism of cellular immunity participates in the prevention or recurrence of EIA or in immunity against the disease, administration of an agent which causes a decrease in the number of lymphocytes or the cells controlling this mechanism of immunity, will induce the recurrence of the disease in horses clinically recovered or suffering from chronic EIA.\textsuperscript{35} Treatment with dexamethasone (DM) is
known to suppress cellular immunity, as well as humoral immunity.\textsuperscript{68,69}

In fact, according to a number of studies, DM treatment causes a remarkable decrease in number of lymphocytes in the circulation\textsuperscript{70-75}, but has no effect on serum antibody titer.\textsuperscript{35}

Kono\textsuperscript{22} injected DM intramuscularly at the dosage of 5 mg/100 lb of body weight, daily for 5 consecutive days into 2 healthy horses, 3 horses suffering from chronic infection, and 4 horses clinically recovered from chronic infection. All the infected horses failed to show a decrease in total leukocyte count of the circulating blood, but manifested a marked decrease of lymphocytes, particularly in lymph nodes, lymphoid follicles of the spleen, and lymphatic apparatus of the intestine. The two healthy controls and one clinically recovered horse presented no symptoms at all, while the other six horses exhibited pyrexia, anemia, the appearance of sideroleukocytes, and viremia as observed at the time of spontaneous recurrence of fever.

Kono \textit{et al.}\textsuperscript{35} carried out a total of 12 experiments with seven infected and three healthy horses. In eight experiments with six infected horses, a rise of body temperature occurred 6 to 8 days after the beginning of DM treatment. A severe fever persisted for 4 to 9 days in five experiments, and a moderate fever for 1 to 2 days in the other three experiments. The remaining infected (one horse) and three healthy horses showed no symptoms. It was also observed that there was no difference in severity of fever or incubation period after the treatment between horses which had remained asymptomatic for 143 to 934 days after the last recurrence, and those which had shown short afebrile periods lasting for one month or less each. In the same experiment, in horses
suffering from fever the virus appeared in the serum immediately before or simultaneously with a febrile attack, and disappeared soon after pyretolysis. No virus could be recovered from one infected horse not manifesting EIA clinical signs, and from any of the three healthy horses. These results would indicate that the response to DM treatment varies in horses chronically infected with EIAV and that the depression of the cell mediated immune system leads to recurrence of EIA; therefore, treatment of EIAV infected horses with corticosteroids should be avoided, whereas immunosuppressive drugs may be useful as a research tool in the generation of possible EIAV variants in chronically EIAV infected horses.

TITRATION OF EQUINE INFECTIOUS ANEMIA VIRUS

The study of EIA was greatly hampered for a long period of time, as the only way to demonstrate the etiological agent of the disease was by horse inoculation. Nevertheless, since 1955 Kobayashi et al. began examining the possibility of propagation of EIAV in various tissue cultures originated from the horse. They reported that of the tissue cultures studied, bone marrow cell cultures and leukocyte cultures were successfully used for the propagation and passage of EIAV. The same authors also found that EIAV could be propagated in horse leukocyte culture, showing a sort of cytopathogenic effect (CPE), and that a strain of this virus which had been adapted to this type of cells could be assayed by using CPE as an indicator; however, they also pointed out that it took a long time to quantitate EIAV by using this type of cells, and that is was not always easy to keep horse leukocyte culture in a satisfactory state for a long period of time. After this report, many
other studies have been conducted about the growth characteristics of EIAV in horse leukocyte culture, including the changes in pathogenicity of EIAV through passages in these cells. Attempts were also made to grow EIAV in a variety of equine cell cultures and in cells from animals of different species including chick embryo, Hela cells and BHK cells, as well as human fetal lung fibroblast. Furthermore, avirulent strains of EIAV have been grown without the induction of CPE in fetal equine kidney cells and in an equine dermal cell line (ATCC-CCL 57).

Equine infectious anemia virus has been titrated in horse leukocyte culture using CPE as an indicator, but as horse leukocytes show spontaneous degeneration it is necessary to titrate the virus by the second step or the third step passage method, which makes the method difficult and tedious to perform. On the other hand, the system is not satisfactory because it is very difficult to find a suitable donor for the leukocytes, and an acceptable bovine serum to be used in the culture medium. Amborski et al. described a simple technique to titrate the infectivity of the cell-adapted strain of EIA in the equine dermal cell line (ED) but the results are reported as variable, therefore, the procedure is not completely satisfactory either. Equine infectious anemia virus has been also quantitated in vivo by injecting the infected material (blood, serum, plasma) into susceptible horses; but although simple and accurate, the procedure cannot be used on a routine basis because it is expensive and time consuming.
The present study was conducted to (i) study the effect of serial passage on the virulence and in vitro growth of a cell adapted strain of EIAV; (ii) generate EIAV variant candidates for further characterization and biochemical comparison; and (iii) contribute to the development of immunoprophylactic protocols against EIAV infection.

MATERIALS AND METHODS

PONIES - Sixteen 2- to 4-year-old Shetland ponies negative for EIAV antibody by the AGID test, were used to carry out the sequential passage of the cell adapted strain, as shown in Table 1. The ponies were stabled in separate stalls screened to exclude blood feeding insects. The rectal temperature of each pony was recorded daily, and the AGID test was performed on the recipient's serum collected three times/week, until the first positive test was confirmed. In addition, nine more ponies were used for the in vivo titration of plasma samples from ponies No. 82, 91, and 64, collected at the first febrile episode after inoculation; accordingly, ponies No. 12, 99, and 111 were recipients of plasma from pony No. 82. No. 217, 112, and 122 from pony No. 91, and ponies No. 94, 114, and 89 were recipients of plasma from pony No. 64. Furthermore, two more ponies, No. 140 and 100, were intravenously (IV) inoculated with 1 ml of a $10^{-2}$ dilution of plasma from pony No. 95, from which no virus was isolated after repeated attempts at cell culture. Finally, one more pony, No. 135, was IV inoculated with 250 mls of whole blood from pony No. 95 to demonstrate the EIAV carrier status of the donor.
VIRUS - The stock of equine cell-adapted strain of EIAV used in this study, was obtained from Dr. W. Malmquist in 1974, and subsequently passaged 109 times by Dr. C. J. Issel, Department of Veterinary Science, L.S.U. in equine dermal (ED) cells and stored at -80°C.

INOCULATION PROCEDURE - The procedure utilized to carry out the sequential passages of the cell-adapted strain of EIAV is depicted in Table 1. The table shows the donor(s), source, and infectivity of the inoculum material. The intravenous route was used in all cases.

After the ponies were inoculated, their rectal temperatures were recorded daily for at least 300 days. When a febrile response was detected ($\geq 39.2°C$), 100 ml of heparinized blood was collected in 50 ml plastic tubes, the plasma separated by centrifugation at 1000 RPM for 10 minutes, and stored at $-80°C$ in 1 ml aliquots and in 50 ml glass tubes. The pony was then closely monitored for clinical signs of disease. Another blood sample was collected during afebrile periods, about 20 days after the febrile attack for harvesting plasma and serum, for further virus isolation attempts, for comparative titrations, and for serum neutralization tests. Serum, and plasma were stored at $-80°C$ until tested.

EVALUATING THE VIRULENCE OF EIAV ISOLATES - A grading scheme was used to evaluate the virulence of the EIAV "strains" generated by serial passage of the cell-adapted strain of EIAV in ponies, as follows. Numerical point values were assigned according to the number of days since the pony was inoculated and the initial febrile episode was observed, i.e., 5 points were given if $\leq 15$ days, 4 points if 16 to 50
days, 3 points if 51 to 100 days, 2 points if 101 to 150 days, and 1 point if 151 to 200 days. In addition, 0.5 point was given for each peak of fever occurring during the entire period of observation. Similarly, 12 points were given if the pony died within 10 days of illness, 8 points if between 11 to 20 days, 6 points if died between 21 to 30 days, 4 points if between 31 to 40, and 2 points if the course of the disease was between 41 and 50 days. Besides, the time the animal lived after inoculation was also taken in account, and as a result 15 points were assigned if the pony lived 20 days after inoculation, 12 points if 21 to 50 days, 8 points if 51 to 100, 6 points if 101 to 150, and 2 points if 151 to 200. Finally, 0.5 point was given per each clinical sign exhibited during a febrile relapse. All observations of recipient ponies receiving DM treatments were not tallied in the virulence grading scheme.

DEXAMETHASONE ADMINISTRATION - The immunosuppressant dexamethasone (DM)\(^b\) was given to pony No. 47 (1st passage) on 200 PID to induce EIA disease as it had remained afebrile for the specified time, and to ponies No. 104 and 101 (2nd passage) which remained afebrile for 180 days post-inoculation. In addition, pony No. 86 (2nd passage) was given DM at two separated occasions, one after the first febrile episode and a quiescent period of 151 days, and the second after its second febrile episode and an asymptomatic period of 228 days. Pony No. 96 (3rd passage) was also given DM at 405 PID, which was 165 days after its 4th febrile episode, to induce the recrudescence of EIA. Pony No. 95 (2nd passage) served as control (no DM). The DM dosage was 0.11 mg/kg of body weight daily for 5 consecutive days, by the intravenous route.
IMMUNOSUPPRESSANT EFFECT OF DEXAMETHASONE - Ten mls of heparinized whole blood were collected from ponies No. 86, 101, 104, and 95 (control) prior to the administration of the first dose of DM, on the day following the last dose, and finally, on the 10th day after the first DM injection was given. The total white blood cell count per microliter (μl), the total number of lymphocytes/μl, and the percentage of lymphocytes were determined by standard procedures. In addition, lymphocytes were separated by the Histopaque-1077 procedure and counted according to a technique that is described elsewhere.\(^7\) The lymphocyte subpopulations were also isolated (separation of T and B-lymphocytes) by nylon wool columns, according to standard procedures\(^67-69\), and the results were given as T and B-cells percentages.

Lymphocytes separated in the previous steps were subjected to transformation by the technique described by Oppenheimer\(^7\) and modified by Barta et al.\(^68\) The lymphocyte layers were treated with three different mitogens at the following levels: (i) Phytohemagglutinin\(^d\) (100, 50, or 25 μg/well; (ii) Concanavalin A\(^e\) (100, 50, or μg/well; and (iii) Pokeweed mitogen\(^c\), 6 μg/well. After 4 to 5 days incubation, tritiated thymidine\(^f\) was added at the doses of 5 μl/well (containing 1 microCi) \(^3\)H-thymidine solution in RPMI 1640. The cultures were reincubated for 18 hours at 37°C in a humidified atmosphere containing 5% CO\(_2\). The samples were then harvested with filter mats, which were then dried in the oven for 30 minutes at 60°C. Finally, the radioactivity was counted in a beta scintillation counter, allowing the vials to be counted for 5 minutes maximum. The results were given as a stimulation index (SI) which is obtained by dividing the counts per
minute (CPM) of stimulated cells by CPM of unstimulated cells from the same individual.

CLINICAL PATHOLOGY - Blood samples from 7 healthy, clinically normal 2 to 4 year old Shetland ponies, which were negative for antibody against EIAV in the AGID test, were analyzed to determine a normal mean value for hemoglobin concentration, packed cell volume (PCV), blood platelet count, and total white blood cell count (TWBC). The purpose was to have a base line for comparison with the same blood components from the chronically EIAV infected ponies involved in this study. Accordingly, samples of blood were obtained one day after some of the febrile recurrences from ponies No.82, 91, 90, 88, 64, and 117, for analysis of the aforementioned blood values. The analysis were carried out by standard procedures at the Clinical Pathology Laboratory, at the School of Veterinary Medicine, Louisiana State University.

POST-MORTEM EXAMINATION - A detailed necropsy was carried out for ponies No.47 (1st passage), 82 (2nd passage), 91, 90, 88, and 127 (3rd passages), 53 (4th passage), and 117 (6th passage), in cooperation with the Department of Veterinary Pathology, School of Veterinary Medicine, and the Louisiana Veterinary Medical Diagnostic Laboratory, Louisiana Department of Agriculture and the School of Veterinary Medicine, in Baton Rouge, Louisiana. Gross and microscopic lesions were recorded and related to clinic history and virus passage.

CELL CULTURES - Fetal equine kidney (FEK) cells prepared at the Department of Veterinary Science, LSU, were used as substrate to test for EIAV in plasma samples collected during and between sequential peaks of fever. The FEK cells were used in preference to ED cells because of
marked increase in the production of EIAV cell-adapted strains in early passed FEK cells according to the author's previous findings. The cells were grown in plastic disposable tissue culture flasks (25 cm\(^2\) growth area). A plasma volume of 0.5 ml was added to each duplicate cell culture flask, which had been drained of media. One hour at 37°C was allowed for viral adsorption. The monolayer was washed off twice with phosphate-buffered saline solution (PBS) pH 7.4, and 5 ml of media was added to refeed the cell monolayers. The cultures were incubated at 37°C for 2 weeks. The cultures were maintained with Eagles minimal essential medium, 25 mM Hepes; 10 mM sodium bicarbonate, (MEM) supplemented with 3% bovine fetal serum, plus 100 μg of streptomycin and 100 units of penicillin/ml. After two weeks incubation at 37°C, the culture fluids were harvested, clarified, and processed as previously described for EIAV antigen in the AGID test. Tests for antigen detection were repeated weekly for up to 12 weeks.

TITRATION OF VIRUS ISOLATES - Plasma samples from 38 selected febrile and afebrile periods were titrated in FEK cells to determine the amount of EIAV present. The results were expressed in terms of tissue culture infectious doses 50% of EIAV (log\(_{10}\) ) per 0.5 ml of plasma and are depicted in Figures 1 to 4.

The plasmas were diluted in ten-fold steps in MEM supplemented with 10% bovine fetal serum, then seeded on FEK cells in duplicate flasks in the amount of 0.5 ml/flask. The flasks were incubated for 60 minutes at 37°C, the plasma excess was removed, the monolayer washed off with 5 mls of PBS, and the cultures refed with MEM containing 3% bovine fetal serum, and finally incubated at 37°C for 2 weeks. After this incubation
the cultures were refed and the harvested supernatants were clarified, and the material processed by the ELISA technique, and by the AGID in some cases, to detect viral p26 antigen.

The ELISA technique, a modified double antibody sandwich technique, was performed by Dr. Barbara Shane from the Department of Veterinary Science, L.S.U. The technique is briefly outlined as follows: (i) The wells of the plates \(^1\) were each coated with 100 ng of horse IgG, which had been isolated and separated from the p26 reference antiserum by \(\text{NH}_4\text{SO}_4\) precipitation and sephacryl column chromatography. After coating, the plates were stored in the refrigerator for 40 hours. (ii) The wells were washed with PBS-Tween \(^3\) 3 times and then 200 \(\mu\)l of test material (cell cultures supernatants) was added, followed by the addition of 5 \(\mu\)l of NP40 \(^k\) (a detergent). This step was followed by an incubation of 2 hours at 37°C. (iii) After the wells were washed 3 times with PBS-Tween 200 \(\mu\)l of a 1:2500 dilution of rabbit anti-p26 serum (antibody against EIAV p26 antigen produced in our laboratory) was added to each well followed by an incubation of 2 hours at 37°C. (iv) The wells were rinsed 3 times with PBS-Tween and goat anti rabbit alkaline phosphatase \(^c\) (antibody is prepared from goat anti rabbit IgG) diluted 1:1000 and in the amount of 200 \(\mu\)l was added, and the plates incubated at 37°C for 3 hours. (v) After this incubation, the wells were washed 3 times with PBS-Tween and the substrate \(^c\) (p-Nitrophenyl Phosphate, Disodium) 200 \(\mu\)g/well added, and the plates were incubated for 45 minutes at 37°C. (vi) Finally, the reaction was stopped with the addition of 50 \(\mu\)l of NaOH(2N), and the plates read in a photometer \(^1\). Adsorbance readings of 0.02 or less were found in
uninfected tissue culture fluids, while readings of 0.1 or higher were regarded as positive for the presence of EIAV antigen (p26). Known dilutions of EIAV p26 as well as negative supernatants fluids and PBS, were included as controls.

**IN VIVO TITRATION OF EIAV** - The plasma samples collected during the first peak of fever of ponies No. 82, 91, and 64 were titrated *in vivo* as follows: one ml of the plasma dilutions $10^{-1}$, $10^{-3}$, and $10^{-5}$ respectively, was IV inoculated into susceptible ponies, according to the following scheme: ponies No. 12, 99, and 111 recipients of pony No. 82; ponies No. 217, 112, and 122 recipients of pony No. 91; and ponies No. 94, 114, and 89 recipients of pony No. 64. After inoculation the rectal temperature of the ponies was recorded daily and 10 mls of whole blood were collected every other day starting on the 5th PID, until the animal became AGID test positive. The plasma infectivity was thus estimated in this manner.

**CROSS SERUM-NEUTRALIZATION** - The cross serum-neutralization test was carried out by the virus dilution-constant serum method in disposable tissue culture flasks. The stock material of each isolate was prepared as follows: plasma known to contain the virus isolate was seeded onto FEK cells in ten-fold dilutions in duplicate flasks to determine infectivity titer. When the titration end point was stable, the supernatant from the end point was clarified and seeded onto fresh primary FEK cells in duplicate flasks for 4 to 5 days. Then the cells were split and reseeded in larger flasks (75 cm² of growth area) and finally transferred to roller bottles (850 cm² of growth area). Finally, after EIAV antigen was detected in the supernatants by the
ELISA technique, the supernatants of the cell cultures were clarified and frozen in 1 ml aliquots, held at ≤ 70°C and tested for EIAV infectivity. The virus stocks used in the serum neutralization test had an EIAV infectivity of $10^{4.5}$ to $10^{6.5}$ TCID$_{50}$/0.5 ml. Sera to be tested for antibody content were inactivated at 56°C for 30 minutes before mixing with the virus dilution. The virus isolates were diluted in ten-fold dilutions from $10^{-1}$ to $10^{-6}$ in cold MEM supplemented with 10% FCS. Then, equal amounts of virus dilution and undiluted serum were mixed and allowed to react for one hour at 37°C. After incubation, 0.5 ml of the mixture was inoculated onto FEK cells in duplicate flasks. As controls, the virus dilutions were mixed and incubated with FCS, and seeded onto FEK cells in duplicate flasks. Duplicate flasks of uninfected FEK cells incubated with a negative horse serum (containing no antibody against EIAV) were included. After the incubation period each flask was rinsed with PBS to eliminate excess virus. The cells were refed with fresh maintenance medium and kept at 37°C for two weeks. After this incubation period the supernatant fluid from each flask was harvested, clarified, and weekly tested by the AGID test to detect EIAV antigen according to the procedure described by Amborski et al.\(^6\)

Antigen prepared from the spleen of a horse with acute equine infectious anemia (007 of 06/21/76) and serum from a chronically infected mare (Lady) were used as reagents for the AGID test. Serum neutralizing activity was expressed as a $\log_{10}$ neutralizing index, calculated as the difference in $\log_{10}$ of the titer of the virus samples with or without test serum. A neutralizing index of ≥2.0 was regarded as positive (neutralization).
RESULTS

FIRST PASSAGE - Two ponies (No. 47 and 80) were inoculated as the first virus passage (Table 2). Pony 80 was euthanized on 101 PID, because of progressive pneumonia after acute equine influenza. This pony became AGID test positive at 18 PID and did not show any clinical sign during the time it was under observation, other than fever and respiratory signs coincident with the equine influenza.

Pony No. 47 became AGID test positive on 20 PID and did not have any febrile attacks for 200 days. The pony was then given DM; it developed clinical signs compatible with EIA 41 days after the 5th (last) dose (Figure 1). Clinical signs compatible with EIA included fever, anorexia, drowsiness, pale mucous membranes, severe CNS depression, diarrhea, weakness, and loss of weight (Table 3). This initial febrile episode lasted for 10 days with 8 subsequent days with normal temperature, followed by a second peak of fever with the aforementioned clinical signs (Figure 1). The pony finally died after 16 days of illness. Five virus isolates were derived from pony No. 47 (Table 4). Two were isolated from samples collected before the pony became AGID test positive. Each of these plasma samples had an infectivity of $10^{1.5}$ TCID$_{50}$ EIAV/0.5 ml plasma. One was isolated from each peak of fever (the infectivity of the plasma from the first peak of fever was $10^{3.5}$ TCID$_{50}$ EIAV/0.5 ml plasma). The fifth virus isolate came from the plasma sample collected between the two peaks of fever.

SECOND PASSAGE - Five ponies were used for the second virus passage. Pony 82 became AGID test positive on 22 PID and showed the initial peak of fever on PID 106 (Table 2; Figure 2). Clinical signs
compatible with EIA fever, anorexia, dullness, CNS depression, and profuse sweating were present for 2 days (Table 3). Subsequently this pony had 5 febrile attacks, but during the 2nd, 3rd, and 4th episodes no other clinical signs were observed. However, in the 5th and the 6th febrile episodes, there was severe disease requiring euthanasia at 358 PID. Virus was isolated from 11 separate plasma preparations from pony No. 82 (Table 4). The plasmas collected at the peaks of fever had an infectivity of \( \geq 10^{1.5} \) TCID\(_{50}/0.5\) ml, with the exception of the plasma from the fourth peak of fever which had an infectivity titer of 10\(^{0.5} \) TCID\(_{50}/0.5\) ml. The highest plasma infectivity was 10\(^{4.5} \) TCID\(_{50}/0.5\) ml, while plasmas collected at afebrile stages had infectivity titers ranging between 10\(^{0.5} \) to 10\(^{1.5} \) TCID\(_{50}/0.5\) ml.

Ponies No. 104 and 95 became AGID-test positive on 22 PID, and pony No. 101 on 24 PID, but in contrast to pony No. 82, none showed clinical signs of EIA through 180 PID. Ponies No. 101 and 104 were given DM at 180 PID and pony No. 95 served as a control. Febrile episodes were noted after the second daily dose of DM had been given to pony No. 101 and 5 days after the 5th dose in pony No. 104. Yet, clinical signs of other than the increase in rectal temperature were not observed (Table 3). On the other hand, pony No. 95 remained afebrile (Table 5). One virus was isolated from plasma collected during the DM induced febrile relapse of pony No. 104, which had an infectivity titer of \( \leq 10^{0.5} \) TCID\(_{50}\) EIAV/0.5 ml of plasma. The pony then experienced two febrile relapses from which two viruses were isolated. In addition, two viruses were isolated from afebrile stages (Table 4). No virus was isolated from pony No. 101, which experienced a second febrile attack 47 days after the first, accompanied by CNS depression and anorexia (Table 3).
Pony No. 95, on the other hand, showed an increase in rectal temperature on 235 PID, but no virus was isolated from the plasma collected at that opportunity, and virus was not isolated from the plasma obtained 3 weeks later. Therefore, two ponies (No. 100 and 140) were IV inoculated with 1 ml of a $10^{-2}$ dilution of the plasmas collected at the peak of fever, and at the afebrile stage following it. Neither of these two ponies experienced a febrile episode or became AGID test positive during 100 PID. Consequently, pony No. 135 was IV inoculated with 250 ml of whole blood from pony No. 95. The recipient pony became AGID test positive on 24 PID.

Pony 86 became AGID test positive on 25 PID and had fever initially on 133 PID. The second peak of fever appeared 41 days later, and the horse remained afebrile for the following 151 days (Figure 2). A short-lived febrile episode was observed with the 3rd dose of DM (Tables 2 and 5), but other signs of EIA were not observed. EIAV was isolated from each one of the three febrile episodes, but virus was not isolated from plasmas collected after the 1st and the 2nd peaks of fever. A virus was isolated from a sample collected at an afebrile stage following the 3rd febrile relapse (Table 4). The plasma collected at the 1st febrile response had a titer of $10^{3.5} \text{TCID}_{50}$ EIAV/0.5 ml. In addition, pony No. 86 was given a second DM treatment on 550 PID (228 days after its last febrile episode, Table 5), but no clinical signs were observed within 30 days of treatment.

THIRD PASSAGE - The recipients of the 3rd virus passage became AGID test positive between 14 and 20 days after inoculation (Table 2). Pony 91 had six febrile episodes, the initial one appearing on 12 PID. Two
more febrile attacks occurred within 60 days, but 138 days elapsed between the 3rd and the 4th peaks of fever (Figure 3). Dullness, CNS depression and anorexia accompanied the last three peaks of fever (Table 3), and at the same time certain hematologic changes were seen (Table 6). Virus was demonstrated and titrated from plasma collected during all 6 febrile episodes and from 3 afebrile samplings (Figure 3). The 1st and the 2nd febrile episodes of pony No. 90 were followed by a rapid sequence of febrile recurrences (Table 2; Figure 3) making it impossible to collect the blood samples during defined afebrile periods. Fever was the only clinical sign observed during the first three febrile responses. In contrast, the 4th and the 5th febrile attacks were accompanied by deep CNS depression, anemia, anorexia, loss of weight, and diarrhea (which lasted for 10 days during the 4th episode). The changes in hematological values of pony No. 90 are shown in Table 6, and details about virus isolation are shown in Table 4. The infectivity of the plasma collected at the first febrile response was $10^{3.5}$ TCID$_{50}$ EIAV/0.5 ml. Pony No. 88 suffered seven febrile attacks, the first one occurring on 14 PID, but 92 days elapsed between the first and the second febrile attacks. However, the following five febrile episodes occurred within 85 days, and caused a profound deterioration of the animal, which led to its euthanasia. The first three peaks of fever of pony No. 88 were not accompanied by EIA clinical signs, while the remainder were accompanied by CNS depression, anemia, anorexia, loss of weight, and weakness (Table 3). Details concerning febrile relapses are given in Figure 3, and details about virus isolation and hematological values from pony No. 88 are shown in Tables 3 and 6 respectively.
Pony 96 also had a peak of fever on 13 PID, and then became AGID test positive. Unlike the other third virus passages recipients, pony No. 96 remained afebrile for 183 days after its first febrile episode, which was followed by two more rectal temperature increases separated by 23 and 26 days respectively (Figure 3). No clinical signs accompanied the first three peaks of fever, but the fourth was accompanied by CNS depression, anorexia, anemia, and weakness (Table 3). Furthermore, pony No. 96 was also given DM on 410 PID (165 days after the fourth peak of fever), but no increase of rectal temperature or any other clinical sign of EIA followed the DM treatment (Table 5). Finally, EIAV was isolated from plasma collected at the first peak of fever (10^{3.5} TCID_{50} EIAV/0.5 ml plasma), but virus was not isolated either from the samples collected at the other febrile relapses or from the material collected at afebrile stages. Details about duration of febrile episodes are shown in Table 2 and Figure 3, clinical signs in Table 3, and virus isolation in Table 4.

Pony No. 120 had the first febrile attack on 12 PID and became AGID test positive two days later. Then two febrile relapses occurred within 36 days and the pony died on 63 PID, after 16 days of clinical disease. The first two peaks of fever were accompanied by anorexia, CNS depression, and weakness, while the third febrile episode also included anemia and diarrhea (Table 3). The sequence of febrile relapses and the titer of EIAV in the plasma collected at the first febrile attack are shown in Figure 3. Pony No. 127 experienced the first febrile attack on 13 PID and became AGID test positive on 17 PID. The pony suffered a total of 5 febrile episodes, the last one being fatal. The first febrile relapse was accompanied by CNS depression, anorexia, and
weakness, while no signs of EIA were observed during the second febrile episode. The third and the fourth febrile attacks were accompanied by CNS depression, but appetite remained. During the fifth febrile episode, CNS depression, anemia, loss of weight, weakness, and diarrhea were present, but appetite remained until the last days of illness. The pony died after 15 days of clinical disease. Virus was demonstrated in 8 samples from this pony, and all eight samples were titrated (Table 4; Figure 3).

FOURTH PASSAGE - The initial febrile episode was observed on 14 PID in pony No. 53. A second peak occurred 39 days later, and altogether 6 febrile episodes were observed (Figure 4; Table 2). Clinical signs of EIA were not seen during the first through fifth febrile episodes, but the sixth episode was accompanied by anorexia, CNS depression, dullness, weakness, diarrhea, and edema of ventral body parts (Table 3). A total of 7 viruses were isolated from pony 53, and the plasma infectivity at the first febrile attack was found to be $10^{3.5}$ TCID$_{50}$ of EIAV/0.5 ml of plasma.

FIFTH PASSAGE - Pony No. 64 used as the fifth virus passage recipient showed the initial peak of fever at 14 PID. Six more peaks of fever occurred (Table 2), but only the fourth, fifth, and sixth febrile episodes were accompanied by CNS depression, anemia, anorexia, loss of weight, and weakness (Table 3). The adverse hematological changes after the second febrile episode gradually increased (Table 6). This pony died on 516 PID and had been under close observation during 300 days after inoculation. Details concerning the sequential febrile episodes are shown in Figure 4. Plasma collected at the first febrile episode
from pony No. 64 was titrated and found to have an infectivity of $10^{4.5}$ TCID$_{50}$ of EIAV/0.5 ml of plasma.

SIXTH PASSAGE - Pony No. 117 served as the sixth virus passage, and had the first febrile relapse on PID 11 and then became AGID test positive on PID 18. The pony's temperature did not return to normal levels, and the horse died on 24 PID, after a clinical disease of 13 days duration. The information concerning EIA clinical signs, virus isolation, virus titer, and hematological values are presented in Tables 2 and 4, in Figure 4, and in Table 6, respectively.

In summary, the first passage recipient (pony No. 47) remained afebrile during 246 days after inoculation and when the animal came down with the disease it was striking and fatal, giving no opportunity for recovery of illness, whereas the second passage recipient ponies experienced the first febrile episode between 106 and 227 PID (two of them were DM induced) and experienced between one and six peaks of fever during at least 300 days period observation postinoculation. The ponies suffered an average of one febrile episode every 86 days. The third passage recipient ponies, on the other hand, suffered the first febrile attack at 12 or 13 PID (Table 2), and experienced from 3 to 7 febrile attacks during the time they were under observation. They had, on an average, a febrile attack every 36 days. Most of the third virus passage recipient ponies died before 200 PID, while all the ponies from the second passage lived for more than 300 days. The fourth passage recipient (pony No. 53) showed the first febrile attack on 14 PID, experienced 6 peaks of fever (one every 33 days, on the average), and died due to EIA after 8 days of severe illness. The fifth passage
recipient (pony No. 64), also had the first febrile episode on 14 PID, experienced 7 febrile episodes during the 300 days period of observation, but died 516 PID. As previously mentioned, the fourth, fifth, and sixth febrile episodes of pony No. 64 were accompanied by severe clinical signs of EIA (Table 3), but the seventh (occurred on 235 PID) was characterized by a rectal temperature increase only. On the contrary, the sixth passage recipient pony (No. 117) had the first febrile attack on 11 PID, and was continually sick for 13 days, exhibiting all the clinical signs of EIA, except the ventral edema of body parts.

In Table 7, a classification is presented according to the type of EIA disease the inoculated ponies showed after inoculation and during the entire period of observation. Accordingly, pony No. 117 (sixth virus passage) suffered an acute form of the disease, while the first virus passage recipient (pony No. 47), one of the second passage recipient ponies (No. 82), five of the third virus passage recipients (Nos. 90, 91, 88, 120, and 127), as well as the fourth virus passage recipients (pony No. 53) and the fifth virus passage recipients (pony No. 64) suffered a type of disease characterized by its chronicity, with several and frequent febrile relapses, and a short episode of severe illness with fatal ending (subacute type). Only one of the ponies (No. 96, a third virus passage) underwent a chronic form of the disease with frequent febrile relapses with no visible clinical signs of EIA, while four out of the 5 second passage ponies (No. 86, 104, 101, and 95) suffered a chronic form of the disease, with the occurrence of sporadic
febrile relapses, but without observable manifestation of clinical EIA (inapparent).

The grading system utilized to evaluate the virulence of the virus strains generated by the serial passage of the ED cell-adapted strain in ponies was not applied to the observations of DM treated ponies following DM treatment in order to avoid a bias in the final results. Therefore, the results obtained were as follows: 0 points for the first virus passage; 4.5 points for the virus second passage; 26 points for the third; 26.5 points for the fourth; 23 points for the fifth; and 28 points for the sixth virus passage.

**ISOLATION OF EIAV FROM FEBRILE AND AFEBRILE PERIODS** - Equine infectious anemia virus isolates were made from 90% of the plasma preparations from material collected during a peak of fever, and from 51% of plasmas collected at afebrile stages (either between two peaks of fever, after a peak of fever, or before the animal became AGID test positive). Equine infectious anemia virus antigen was first detected by the AGID test, at approximately the fifth week after seeding the plasmas onto FEK cells, the time varying from 2 to 11 weeks. There was no difference in the time required to detect the antigen from a sample collected at the peak of fever or at an afebrile stage, and CPE was not observed in the EIAV infected FEK cells.

**EFFECT OF DEXAMETHASONE ADMINISTRATION** - Pony No. 47 (first passage) was given DM on 200 PID as no clinical signs of EIA had occurred. There was no detectable clinical response that was temporally associated with this immunosuppressive treatment. In contrast, ponies No. 86, 104, and 101 (second passages) which were also
given DM, did have short lived febrile responses, but they were not accompanied by other clinical signs. Virus was demonstrated in plasmas from the DM induced febrile episodes from ponies No. 86 and 104, but not from plasma collected from pony No. 101. Pony No. 86 received a second treatment with DM on 550 PID and (228 days after the last febrile response), but with negative results. Finally, pony 96 (3rd passage) was given DM on 410 PID after suffering 4 febrile attacks 165 days after the last febrile episode, but with negative results (Table 5).

IMMUNOSUPPRESSANT EFFECT OF DEXAMETHASONE - Whole heparinized blood was collected from ponies No. 86, 101, 104 and 95 (second passages) at three occasions: before the first DM administration, on the day following the last DM doses, and on day 10 after the first DM injection. Total white blood cell counts (TWBC) and the percentage of lymphocytes were determined for each one of the samples collected (Table 8). The results show that the TWBC was reduced by $3 \times 10^3 / \mu l$ at the second count of pony 86, but the counts returned to about the initial value at the third count, while ponies No. 101 and 104 showed a slight increase in the TWBC in the second and the third counts (compared with the first count), and a similar result was found for pony No. 95 (control). As an average, the reduction of lymphocytes was of 20% and 25% at the second and third counts respectively, in the DM treated ponies, while in the control pony a reduction of 17% was found in the second count, but the third count was higher than the first. However, the response of the DM treated ponies was very variable as can be inferred from Table 8. Lymphocytes as a percentage were lower in the third count, compared to the first one in all the treated ponies.
Lymphocytes of the same ponies were separated from the rest of the blood components, counted, and classified into T and B-cells, before DM administration, on the day following the last dose of DM, and on day 10 after the first DM injection (Table 9). It was found that lymphocytes decreased by 52% in the second count of pony No. 86, but the count returned to pretreatment levels at the third count; while for pony No. 104, the second count showed a decrease of 2.5%, and of 29.5% in the third count; however, lymphocytes of pony No. 101 were reduced by one third at both counts, compared with the pretreatment count. On the contrary, lymphocytes of the control pony were slightly increased at the second and third counts. T-cell counts on the other hand, were reduced by 17% in the second and third counts of ponies No. 86 and 101, while the decrease for pony No. 104 was negligible, and counts for control pony were equal to pretreatment counts. B-cells were reduced by 25% in the second count of ponies No. 86 and 104, but returned to pretreatment levels at the third counts, yet, for pony No. 104 B-cells were moderately reduced at the second count (4.3%), but decreased by 39.3% at the third count. B-cell counts of control pony remained substantially unchanged in the three counts.

The information concerning the results of the lymphocyte transformation test, carried out for lymphocytes from ponies No. 86, 101, 104 (DM treated) and pony No. 95 (control), are shown in Table 10 (stimulation indexes). From these results, it seems that there was no effect of the DM treatment on the lymphocyte transformation (blastogenesis) in vitro, using the three mitogens we used at different doses, i.e., PHA, Con A, and Pokeweed mitogen. Finally, it seems that
DM treatment did induce the occurrence of a febrile episode in ponies No. 86, 104, and 101, but not in ponies No. 47, 96, and 86 (in the second treatment).

In summary: (i) DM treatment causes a variable response in the TWBC counts, but the general pattern would be a transient moderate increase, yet, lymphocytes as a percentage tend to decrease by about one third in general terms; (ii) T-cells were reduced by 17% compared to pretreatment counts; (iii) Reduction of B-cells were up to 39%; (iv) the DM treatment did not affect lymphocyte transformation assay in vitro with the mitogens used in this study; and (v) DM treatment did induce febrile episodes of EIA, in some chronically EIAV-infected ponies.

CLINICAL PATHOLOGY - Table 6 shows the hematological values obtained from 6 of the inoculated ponies, from samples taken one day after the beginning of the EIA febrile episodes. It is seen that hemoglobin is below normal values in all ponies after the febrile attack occurred and a steady tendency to decrease was found after each new febrile episode. Consequently, the same trend is found for the packed cell volume (PCV) for which values below 50% of normal are found after several febrile episodes. It seems that after the fifth or sixth febrile episode the PCV is under 20% (Table 5, ponies No. 91, 90, and 88). On the other hand, excepting pony No. 117 (sixth passage) which suffered an acute illness, the blood platelet counts were found extremely diminished (50% of normal values in all the cases), sometimes as low as $0.07 \times 10^5/\mu l$ (pony No. 90 after the fifth febrile episode). Similarly, the TWBC count was found to be below normal in most of the observations, tending to decrease even more with each new febrile
relapse, to reach values as low as $3 \times 10^3 / \mu l$, i.e., one-third of normal.

**POSTMORTEM EXAMINATION.** A detailed necropsy was carried out for ponies No. 117 (acute EIA - death following a short incubation period), No. 47 (first passage), No. 82 (second passage), Nos. 91, 88, 127, 120 (third passage), and No. 53 (fourth passage), which suffered the subacute form of the disease (an initial febrile episode, followed by several febrile relapses and death after about two weeks of severe illness). The only chronic case of EIA was pony No. 96 (third passage) which was still alive at the time this work was written, while two of the horses suffering the inapparent type of EIA were euthanized, but no necropsy was performed.

The most prominent macroscopic and microscopic lesions were found in liver, kidney, spleen, lymph nodes, and bone marrow, while no prominent lesions were found in heart, colon, cerebrum and cerebellum, and no lesions attributable to EIAV were found in pancreas, small intestine, urinary bladder, thyroid, and pituitary gland.

In the acute case of EIA, liver was found to be enlarged, friable and swollen, and at the microscopic examination prominent fatty changes were seen, as well as large number of hemosiderin laden macrophages in the sinusoids, necrotic hepatocytes, and the periportal areas infiltrated by numerous lymphoid elements intermixed with few macrophages and neutrophils. In the subacute cases, the liver was found grossly normal in some cases, but it was usually congested, dark red with an accentuated lobular pattern, while the microscopic lesions were similar to those described for the acute case, with the exception that hemosiderocytes were much more abundant in the subacute cases. The
kidney was macroscopically hemorrhagic in the acute case of EIA with very poorly defined striation in the cortex and medulla, while in the subacute cases, it was found pale or congested, and with petechial or ecchymotic hemorrhages. The microscopic lesions in both types of EIA were similar, mostly hemorrhages scattered through the parenchyma of cortex, vacuolar degeneration in the renal proximal tubular epithelium with dystrophic mineralization, the interstitium increased in prominence with lymphoid infiltration, and increase in neutrophils and macrophages; besides, the glomeruli were found to be hypercellular and tending to fill the Bowman's space, and also, a mild lymphocytic and plasma cell infiltration was found in the glomerular tuft. The spleen on the other hand, was found enlarged and dark red in the acute case, but in some of the subacute cases a normal size spleen was found, while the microscopic examination revealed a highly cellular spleen with splenic corpuscles larger than normal, germinal centers with many mitotic figures, and lack of maturation zones for the lymphocytes in the acute cases. In the subacute cases, lymphocyte infiltration and large areas of necrosis with fibrin and hemorrhages were the predominant lesions. Similarly, the lymph nodes were severely affected in both types of EIA disease, with hyperplasia and hemorrhages being the dominant macroscopic lesion, especially in the acute case, while the microscopic common lesion was hyperplasia with lymphocytic infiltration. The bone marrow showed evidence of regeneration of the hematopoietic tissue in the acute and in the subacute cases of the disease, and the microscopic findings were increased cellularity, large numbers of erythroblasts, prorubricytes, and
metarubricytes, lymphoid and myeloid hyperplasia, abundance of plasma cells, and many macrophages with hemosiderin.

CROSS SERUM-NEUTRALIZATION TEST. The results of the cross serum-neutralization test carried out for 1 virus isolate with 3 antisera collected from pony No. 82 are shown in Table 11. The results show that virus isolate P82-1 (isolated 106 PID) was not neutralized by serum collected on PID 127, but was strongly neutralized by antiseraums collected at a later stage of the disease (280 and 325 PID respectively).

The cross serum-neutralization test carried out for four virus isolates and three antiserums from pony 127 are presented in Table 12. At first it is seen that the serum collected on 31 PID did not contain enough antibody to neutralize the virus isolate 127-1 and did not neutralize any of the other three viruses generated thereafter, while the serum collected at 125 PID did strongly neutralize the two virus isolates previously formed, but weakly neutralized virus isolate P127-3 and failed to neutralize virus isolate P127-4. Finally, the serum collected on 158 PID did neutralize virus isolates P127-1, P127-2, and P127-3, but failed to neutralize virus isolate P127-4. From these results it can be seen that the neutralizing potency of a given serum sample varied when tested against different viruses. Serum collected at 125 PID had the highest neutralizing indexes against virus isolates P127-1 and P127-2, and in a similar fashion, sera collected on 280 and on 325 PID showed a strong neutralizing activity against virus isolate P82-1.
DISCUSSION

Six serial passages of a cell-adapted strain of EIAV were carried out in a total of sixteen 2 to 4 year old Shetland ponies to study possible changes in virulence of this cell-adapted strain, and mainly to isolate EIAV from plasma samples taken at the time of febrile attacks, and also at afebrile stages of the infection. As a result, it was found that the virulence of this cell-adapted strain of EIAV increased through passages in ponies; in addition, a total of 77 isolates of EIAV were made, 55 from plasmas collected at the time of febrile episodes, and 22 from plasmas collected at afebrile stages of the chronically EIAV infected ponies. It was found also that EIAV infectivity was higher for samples drawn at a febrile episode than for samples collected at afebrile stages. Furthermore, virus isolates and sera collected at different times after infection from the same pony proved the antigenic variation of the sequential virus isolates.

The sixteen ponies used for the successive passage of the cell adapted strain of EIAV became AGID test positive between 14 and 25 PID, which is in agreement with previous reports.\textsuperscript{13,18,19} It was found that the time required to detect precipitating antibody was approximately the same in the low and high virus passage ponies. The first febrile response occurred much earlier in the ponies receiving the highest virus passage (third, fourth, fifth, and sixth) than in the recipient ponies of the lower passages of the virus (first and second).

Pony 47, the first EIAV passage recipient had the first febrile episode on 246 PID; then, this pony had the longest incubation period, probably because it was inoculated with an EIAV attenuated through high
passage in cell culture; but despite this, the clinical disease the pony underwent was one of the most severe observed in this study. On the other hand, the response might be independent of the immunosuppressive treatment, as DM induced immunosuppression would last for about one week after the last injection (Table 9). The second passage ponies, on the other hand, experienced the first febrile episode between 106 and 227 PID (two of them DM induced). The recipients of the third, fourth, fifth, and sixth virus passages showed the first febrile attack between 11 and 14 PID, which would indicate an increase in the virulence of this cell-adapted strain. The number of febrile episodes, the course of the disease, the time lived after inoculation, and the EIA clinical signs substantiate the statement that the virulence of the cell-adapted strain of EIAV increases through serial passages in ponies, although individual variation within passages was high. The second passage ponies scored lower in the grading system, which might be due to the infection with an EIAV which probably had not regained virulence, as compared with the pattern of disease in the higher virus passages. The number of febrile episodes experienced by the third passage ponies was similar to observations reported by Kono et al.\textsuperscript{18,19}, but do differ from another report.\textsuperscript{13}

Febrile episodes in most of the ponies lasted from 1 to 5 days, which is in accordance with previous reports.\textsuperscript{11,17-19} However, the first passage recipient suffered two febrile attacks lasting for 10 and 16 days, respectively. The DM induced febrile episodes were short lived. The clinical signs observed in this study are in agreement with reports from other workers.\textsuperscript{5,19} Fever, CNS depression, anemia, loss of
weight, and weakness were the most common EIAV clinical signs observed, while diarrhea was rarely seen, and only in terminal cases. Ventral edema was seen in one of the sick ponies only after about 30 days of continuous illness.

Equine infectious anemia virus was isolated from 90% of the plasma preparations from blood collected at a peak of fever and from 51% of the plasmas collected at afebrile stages. In previous reports, EIAV was isolated in horse leukocyte cultures from serum samples collected at the same stages from chronically infected horses with production of CPE. Isolation of EIAV variants was accomplished at a high rate from plasma seeded in primary FEK cells, but without the induction of CPE. Since the isolation rates were similar for low and high passages, and from initial and final febrile episodes, it is suggested that the mutation of this cell-adapted strain of EIAV allows for its cultivation in FEK cells and is relatively stable.

No virus was isolated from pony No. 95 (2nd passage) or from pony 101 (DM induced, second passage). To demonstrate the carrier status of pony No. 95, two ponies were inoculated with diluted plasma from that pony, but the two recipients remained EIAV susceptible; yet, the pony inoculated with 250 mls of whole blood from pony No. 95 seroconverted to positive in the AGID test on 27 PID. This result supports the concept that once a horse is infected with EIAV, it becomes an EIAV carrier for life. Horses chronically infected with EIAV usually become quiescent and do not show EIA disease clinical signs for long periods of time, but they do have a persistent viremia, regardless of the clinical course. In this case the reasons for the failure
to isolate EIAV from ponies No. 95 and 101 could be a lack of plasma viremia (EIAV present in leukocytes only); hence, the increase in rectal temperature was probably not due to EIAV replication and propagation in the host.

The EIAV variants generated through this study have been used in several experiments concerning EIAV vector transmission. Accordingly, a pony was IV inoculated with 1 ml of plasma prepared from whole blood collected from the peak of fever of pony No. 53 (fourth passage), which had a titer of $10^{3.5}$ TCID$_{50}$ of EIAV/0.5 ml of plasma. The recipient pony showed clinical signs of the disease on 15 PID and 18 days later showed neurological clinical signs which included CNS depression, disorientation, knuckling at the fetlock, hypermetria, kneeling, rolling on its back, and finally paralysis of the hind legs. These neurological clinical signs do differ from those of visna virus affected sheep, as the paralysis usually progresses slowly and the animal remains alert and can survive for several weeks.$^{84}$ In addition, tremors of the head and facial muscles, and blindness are frequently seen.$^{85}$ The cerebro spinal fluid was shown to contain antibody against EIAV, but no virus was isolated in primary FEK cells. EIAV was isolated from brain and spinal cord tissues and the material was found to have an infectivity of $10^{0.5}$ TCID$_{50}$ of EIAV/gm of tissue. The clinical description of this particular case is similar to a report from McIlwraith et al.$^{71}$, but in this case EIAV was isolated and titrated from CNS tissue (the best source for visna virus isolation in sheep)$^{84}$. Therefore, EIAV might play an important role in many clinical cases in which CNS clinical signs are observed.
The infectivity titer of the plasmas from which EIAV was isolated from a peak of fever varied from $\leq 10^{0.5}$ TCID$_{50}$ of EIAV/0.5 ml to $10^{5.5}$ TCID$_{50}$ of EIAV/0.5 ml of plasma, but usually the lowest titer was $10^{2.5}$ TCID$_{50}$ of EIAV/0.5 ml (a titer of $10^{0.5}$ TCID$_{50}$ of EIAV/0.5 ml of plasma was found in one case only). An increase in the EIAV plasma infectivity was not found in sequential virus isolates from the same pony, the results being in agreement with a previous work. On the other hand, the results of the in vivo and in vitro titrations were quite similar suggesting that the quantitation of EIAV can be carried out in primary FEK cells in an accurate manner, thus avoiding the use of other cumbersome techniques.

According to the course of EIA disease the ponies suffered, most of the cases corresponded to the subacute form characterized by the occurrence of several febrile relapses and a fatal ending, which coincides with previous reports. The acute form of the disease seems to be infrequent, and in this case, only pony No. 117 (sixth passage) was stricken by a highly passaged virus which had become very virulent, giving support to the notion that the cell-adapted strain of EIAV increases its virulence through successive passages in ponies. The inapparent form of the disease was more common than the chronic form and was shown by four of the five second passage recipient ponies, which might be an indication that relatively avirulent EIAV tends to be perpetuated in its host, being more stable than the virulent strains, and perhaps in a provirus state (integration of viral nucleic acid with the cell's DNA).
The changes upon blood components indicated that the physical condition of the ponies worsened with successive febrile attacks. The blood platelet reduction was severe and coincident with the febrile attacks, which was consistent with previous reports. The leukopenia that usually increased with progressive febrile attacks might be related to a suppressive effect of EIAV on the immune system of the host as reported for other retroviruses. The anemia observed in the ponies increased after each febrile attack, the hemoglobin and the PCV reaching very low levels (a third of normal in some of the cases). This is in agreement with previous reports.

The response of the chronically infected ponies to the administration of the immunosuppressant DM was inconsistent and unpredictable and was in partial agreement with results from Kono et al. A short lived increase of rectal temperature was found in 3 of 6 ponies treated and EIAV was isolated from 2 of them, but no clinical signs of EIA encompassed the induced febrile episodes. The TWBC increased in 2 of the ponies treated (101 and 104), which is in agreement with some reports, but decreased in one of the ponies. Thus, these results substantiate the statement that DM response is variable among DM treated ponies. Lymphocytes as a percentage were consistently found low after DM treatment as previously reported. Separated lymphocytes and T-cells were found decreased at the second count, but at about pretreatment levels at the third count, results that coincide with previous reports. Finally, B-cells were reduced but in a lesser extent in general terms and no
specific references on the effect of DM treatment on B-cells of chronically EIAV infected ponies were found in the literature reviewed.

The cell-mediated immune system (CMI) is suppressed by repeated IV administration of DM and latent viral infections may also be activated by corticosteroid administration. This is probably because corticosteroids inhibit phagocytosis by macrophages by interfering with antigen recognition, and altering immunologically induced inflammatory reactions. It seems that sustained elevated plasma levels of corticosteroids are necessary for suppression of various lymphocyte functions and that there are differences in the corticosteroid response among individuals of the same species. The cause of lymphopenia induced by corticosteroids is not well known, but it has been found that DM induces irreversible G₁ arrest. Other reports suggest that the lymphopenia is due to a redistribution of lymphocytes from the circulation to other body compartments. Still other reports state that the lymphopenia is due to the destructive effect of corticosteroids on lymphoid tissue. It is possible that by inhibiting protein synthesis, corticosteroids suppress the afferent limb of the immune response.

The results of the lymphocyte transformation assay indicated that there was no effect of DM treatment upon the lymphocyte blastogenesis, a result that does not agree with a report from Tarr et al. who found that DM in horses causes an average of 48% suppression of PHA induced blastogenesis, 43% suppression of Con A induced blastogenesis, and 29% suppression of PWM induced blastogenesis. Magnuson et al. reported that chronic in vivo treatment of ponies with corticosteroids caused a
marked decrease in the absolute number of circulating lymphocytes, but little effect on in vitro PHA lymphocyte stimulation. Similarly, because peripheral blood lymphocytes from ponies appear not to be physically altered by large concentrations of corticosteroids and neither short nor chronic corticosteroid treatment affect the response of ponies to PHA, those authors suggested that the pony should be placed in the category of corticosteroid-resistant animals, along with man, guinea pig, and monkey. It is finally proposed that the apparent unaltered, mitogenic response found in this study is due to a decreased number of suppressor T-lymphocytes in circulation.

The study concerning the macroscopic and microscopic lesions found in acute, subacute, and chronic cases of EIA showed that liver, kidney, and spleen of ponies which died due to acute EIA have marked gross and microscopic lesions of EIA, while in the subacute and inapparent cases those organs may not show macroscopic alterations or they are much less severe than the lesions found in acute cases. While microscopic lesions are severe and constant in the liver and kidney, the spleen may not exhibit prominent lesions. The lymphatic nodes are seriously affected in the three types of EIA, the most severe changes being found in the histopathological study of material from acute cases. The same macroscopic and microscopic lesions were found in the bone marrow of ponies dying due to either type of EIA disease. In addition, the lesions found in the heart of ponies dead by any type of EIA disease were found to be of no importance for the diagnosis of EIA. Macroscopic and microscopic lesions of the colon were found to be different in the acute, subacute, and inapparent types of EIA disease. No macroscopic
lesions of cerebrum and cerebelum were found in subacute and inapparent cases of EIA while congestion was observed in acute cases; otherwise, no microscopic lesions were found in cerebrum and cerebelum of acute cases, but they were reported for chronic cases (slight infiltration of lymphocytes in the meninges). These macroscopic and microscopic lesions are in agreement with previous reports. 11,13

As previously mentioned, a pony for another project was inoculated with a virus isolate generated in this study and developed clinical signs of neurological disease already described and consistent with meningoencephalitis. The macroscopic and microscopic lesions comprised enlargement of the ventricular system, and grey-brown discoloration of ventricular surfaces, but no lesions were found in the spinal cord. The histological lesions observed in the brain were mostly meningitis, choroiditis, granulomatous ependymitis, subependymal encephalitis, and lymphoid infiltration into the subependymal neuropil and a lesser number of reticuloendothelial cells. These macroscopic and microscopic lesions, as well as the clinical signs are in agreement with a previous report from McIlwaraith et al. 71 and occurred in an acute case of EIA. These macroscopic and microscopic lesions are also found in sheep dead due to visna virus infection, but demyelination is a prominent effect in the brain of visna virus affected sheep. 84 In addition, visna virus produces lesions in the spinal cord. These lesions, on becoming confluent, give rise to large lesions which tend to necrose and form cavitations. 86 As pointed out by McClure et al. 87, there is possible a number of cases in horses showing progressive hind-end incoordination due to EIAV damage within the spinal cord. In this case, the clinical
signs point to the brain and are correlated with the macroscopic and microscopic findings in the altered brain structure and tissue.

The results of the cross serum-neutralization test carried out for virus isolate P82-1 (isolated from plasma taken at the first febrile episode of pony No. 82) and 3 antisera collected at different times after inoculation, and for 4 virus isolates and 3 antisera from pony 127, showed that the virus isolates from the same pony are not neutralized by sera collected before their isolation. Sera collected at about 30 days after a febrile episode, however, show neutralizing activity which increases in subsequently collected serum samples. These results are in agreement with previous reports.13,18,19

The fact that a serum collected after a febrile episode neutralizes EIAV previously generated is something expected, as antibody previously formed against previously arisen EIAV strains would be present in the serum; then, as found in this study, a serum collected at a defined time after inoculation, does not contain antibody against EIAV appearing after the serum collection. This result would indicate a partial common antigenicity between the virus isolated one after another in the same pony, but at the same time would indicate an antigenic variation (a subtle change) in the EIAV isolate.

Finally, if the EIAV variants are formed, either under the pressure of the host immune response (antibody and Tc cells mostly)28, or by natural mutation (not induced by external stimulus), in both cases by accumulation of point mutations (that involve a single nucleotide), the host cell mediated immune response would contribute to the propagation
of the new virus variants, as the virus hides in the leukocytes (mostly macrophages) to escape the host immune system.

A work from Parekh et al. showed that EIAV contains four major nonglycosylated proteins (p26, p15, p11, and p9) and two glycoproteins (gp90 and gp45), which together account for greater than 95% of the total virion protein. As the nature of the antigenic variation of EIAV has remained unknown, Parekh et al. undertook the biochemical analysis of virus isolates P82-1 and P82-6 which were obtained in this work, from plasma samples collected at the first and sixth episode of pony No. 82, a virus second passage. The SDS-PAGE revealed different electrophoretic mobilities for the respective virion surface glycoproteins (gp90 and gp45), but identical migration rates for the internal nonglycosylated virion proteins (p26, p15, p11, and p9). Peptide mapping comparisons demonstrated structural variations between gp90 and gp45 components of the P82-1, P82-6, and the prototype cell-adapted EIAV strain, but the identity of the virus variants was maintained in long term culture of the viruses in FEK cells. It was also found that structural alterations seems to be much greater in gp45 compared to gp90, but, in contrast to the glycoproteins of EIAV, no differences were detected among the major nonglycosylated virion proteins p26, p15, p11, and p9, when they were compared by the same procedures. The suggestion is that during replication in the horse, EIAV glycoproteins can undergo detectable structural alterations in the absence of any change in the major internal proteins of the virus. Changes in glycosylated or nonglycosylated proteins were not detected during replication in vitro where immune pressure was absent. The nature of the antigenic variation
in EIAV remains to be determined and it is unknown whether the observed structural alterations occur in amino acid sequence, oligosaccharide structures, or both. Analysis of the glycopeptide fractions by gel filtration procedures may elucidate further, the site(s) of antigenic variation in EIAV. The identification of key antigenic determinants in the glycoproteins of EIAV, and the effects of changes in these regions on serological properties may provide the information necessary for developing effective immunization regimes against EIA disease. Through this study, the required reagents have been collected and prepared to continue the effort to elucidate the case of the antigenic drift in EIAV, and hopefully to obtain an effective vaccine against EIA virus.

SUMMARY

Six serial passages of a cell-adapted strain of EIAV were conducted in Shetland ponies. The 16 recipient ponies became agar-gel immunodiffusion positive by 25 days post-inoculation. The first virus passage recipient had the first febrile response on 246 post-inoculation day (PID), while ponies from the second passage showed the first febrile attack after 100 PID, and some of the febrile responses were dexamethasone induced. On the other hand, ponies from the third through the sixth passage experienced the first febrile response by 13 PID.

The most important clinical signs exhibited by the ponies, along with the EIA episodes were fever, CNS depression, anemia, anorexia, loss of weight and weakness, while diarrhea and ventral edema of the body parts were rarely observed. The subacute type of the disease was predominant, followed by the inapparent type, and only the highest passage pony suffered the acute type of the disease. In addition,
according to the results of a grading scheme prepared to evaluate the virulence of the virus isolates, it was found that the virulence of the cell-adapted strain increased through serial passages in ponies, although individual variations occurred.

Changes upon the blood components were studied from samples taken on the day following some of the febrile attacks, and as a result, hemoglobin, the packed-cell volume, the total count of leukocytes, and the thrombocytes were found under normal levels; in addition, the immunosuppressant dexamethasone (DM) was given to some of the ponies to induce clinical signs of EIA and to study its effect upon white blood cells. It was found that DM induced a short-lived febrile response in three of the five treated ponies, but EIAV was isolated from plasma of two of the ponies only. Besides, it was found that DM treatment induces a transient increase in total white blood cell counts, while T and B cell counts were reduced. In addition, the DM treatment did not affect the lymphocyte transformation in vitro with the mitogens used in this study.

Postmortem examination conducted in ponies which died due to acute and subacute types of EIA, showed that severe hemorrhagic lesions were common in the acute cases, and that the most affected organs in both types of EIA disease were liver, spleen, kidney, lymph nodes, and bone marrow, as derived from the microscopic examination of the affected tissues.

Equine infectious anemia virus was isolated from 90% of the plasmas collected at febrile stages and from 51% of the plasmas collected at afebrile stages. The plasmas were seeded on primary fetal equine cells,
where EIAV grew without the induction of cytopathogenic effect. In addition, the EIAV plasma infectivity was found to be higher for plasma samples collected at the peaks of fever, than from plasmas collected at afebrile stages. Finally, the cross serum neutralization test was carried for one virus isolate from a second passage pony, and for four virus isolates from a third passage pony, with sera collected from the same pony at different times after inoculation. It was found that serum collected a short period of time after virus isolation, did not have any neutralizing activity against the virus isolate, but the neutralizing activity increased in sera collected thereafter. Equally important, a serum collected at a given time displays neutralizing activity against isolates made previously, but not against cloned isolates of EIAV from febrile episodes after the serum collection. This finding supports the suggestion that antigenic variation of EIAV occurs within the horse, and may be the mechanism by which the virus temporarily escapes the host immune response. The mechanism known as "antigenic drift" of EIAV, has hampered the production of an effective vaccine against EIA, and also the establishment of an operative sanitary campaign. It is possible that by the future research on EIA at Louisiana State University, using the reagents generated through this study, the nature of the antigenic variation of EIAV will be disclosed, making possible the production of an immunogen against equine infectious anemia disease.
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to infection. Histogenesis of the lymphoid response and


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Suppression of CMI system by Corticosteroids. 2:129-133,
1978.


FOOTNOTES

b. Schering Corporation. Kenilworth, NJ.
c. Sigma Chemical Company. St Louis, MO 63178, USA.
e. Calbiochem. San Diego, CA.
g. Falcon Plastics, Division of Becton, Dickinson and Co. Oxnard, CA.
h. Gibco Laboratories. Grand Island, NY.
i. Dynatech Laboratories, Inc. 900 Slaters Lane, Alexandria, VA, USA.
k. The Shell Company. Shell Plaza. Houston, TX 77002.
m. Corning Glass Works. Corning, NY.

n. The EIAV isolates are named according to the pony from which the virus was isolated, and the respective febrile relapse; for instance, P82-1 is the EIAV isolate from the first peak of fever of pony 82.

o. Glycoprotein changes associated with antigenic variation during persistent infection by equine infectious anemia virus, a retrovirus. Sent for publication on 01/26/83 to J Biol Chem.
TABLE 1. Inoculation procedure utilized to carry out the sequential passage of cell-adapted strain of EIAV in sixteen Shetland ponies

<table>
<thead>
<tr>
<th>Passage</th>
<th>Donor Source of inoculum</th>
<th>Inoculum infectivity (log₁₀ TCID₅₀/0.5 ml)</th>
<th>Recipient pony number</th>
</tr>
</thead>
<tbody>
<tr>
<td>First</td>
<td>EIAV cell-adapted strain</td>
<td>10⁶</td>
<td>47,80</td>
</tr>
<tr>
<td>Second</td>
<td>Pool of plasmas collected before first AGID positive test (18 and 16 respectively)</td>
<td>10¹.₅</td>
<td>82,104, 95,101</td>
</tr>
<tr>
<td>Second</td>
<td>Plasma collected before first AGID positive test (18)</td>
<td>10¹.₅</td>
<td>86</td>
</tr>
<tr>
<td>Third</td>
<td>Plasma collected at first febrile episode (106)</td>
<td>10⁴.₅</td>
<td>91,90,88 96,120,127</td>
</tr>
<tr>
<td>Fourth</td>
<td>Plasma collected at first febrile episode (12)</td>
<td>10⁵.₅</td>
<td>53</td>
</tr>
<tr>
<td>Fifth</td>
<td>Plasma collected at first febrile episode (14)</td>
<td>10³.₅</td>
<td>64</td>
</tr>
<tr>
<td>Sixth</td>
<td>Plasma collected at first febrile episode (14)</td>
<td>10⁴.₅</td>
<td>117</td>
</tr>
</tbody>
</table>
TABLE 2. Inoculation procedure, appearance and duration of febrile episodes in recipient ponies through six serial passages of a cell-adapted strain of EIAV

<table>
<thead>
<tr>
<th>Passage</th>
<th>Donor</th>
<th>Recipient pony no.</th>
<th>Period of Observation (days)</th>
<th>Appearance of 1st positive (PID)</th>
<th>Appearance of given febrile episodes (PID)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st cell culture</td>
<td>47</td>
<td>291</td>
<td>20</td>
<td>246(1)*</td>
<td>Died on 291 PID</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>101</td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>82</td>
<td>358</td>
<td>22</td>
<td>106(2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>104</td>
<td>300</td>
<td>18</td>
<td>135(2)</td>
<td>195(3)</td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>300</td>
<td>18</td>
<td>214(20)</td>
<td>264(3)</td>
</tr>
<tr>
<td></td>
<td>101</td>
<td>300</td>
<td>22</td>
<td>302(3)</td>
<td></td>
</tr>
<tr>
<td>2nd</td>
<td>Ponies 47 and 80 (pooled plasma)</td>
<td>104</td>
<td>300</td>
<td>22</td>
<td>187*(1)</td>
</tr>
<tr>
<td></td>
<td>86</td>
<td>500</td>
<td>24</td>
<td>227(3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>91</td>
<td>323</td>
<td>19</td>
<td>181*(2)</td>
<td>228(2)</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>157</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>101</td>
<td>300</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd</td>
<td>Pony 82 plasma</td>
<td>88</td>
<td>211</td>
<td>13(3)</td>
<td>174(3)</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>440</td>
<td>20</td>
<td>325*(1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>63</td>
<td>18</td>
<td>12(2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>127</td>
<td>172</td>
<td>14</td>
<td>4(1)</td>
<td></td>
</tr>
<tr>
<td>4th - Pony 91 plasma</td>
<td>53</td>
<td>211</td>
<td>17</td>
<td>13(12)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>300</td>
<td>20</td>
<td>14(2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>25</td>
<td>18</td>
<td>14(5)</td>
<td></td>
</tr>
<tr>
<td>5th - Pony 53 plasma</td>
<td>64</td>
<td>300</td>
<td>20</td>
<td>23(2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>25</td>
<td>18</td>
<td>28(1)</td>
<td></td>
</tr>
<tr>
<td>6th - Pony 64 plasma</td>
<td>117</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* () = Duration of febrile episode in days.
* Decamethasone associated febrile episode.
<table>
<thead>
<tr>
<th>Virus serial passage</th>
<th>Recipient pony no.</th>
<th>Febrile episode no.</th>
<th>Clinical signs</th>
<th>Ventral edema of body parts</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st 47 1,2</td>
<td>1,2</td>
<td>x x x x x x x x x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd 82 2-4 5 6</td>
<td>1 2 4 5 6</td>
<td>x x x x x x x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd 86 1-3 104 101</td>
<td>1 2 95 3</td>
<td>x x x x x x x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4th 91 90 88 96 120 127</td>
<td>1-3 1-3 1-3 1-3 1-2 3 1</td>
<td>x x x x x x x x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5th 53 6 4-6 7</td>
<td>1-5 6 1-3 4-6 7</td>
<td>x x x x x x x x 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6th 117 1</td>
<td>1</td>
<td>x x x x x x x</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE 4. Reisolation success of a cell-adapted strain of EIAV in fetal equine kidney cell cultures from plasma collected during febrile and afebrile periods from inoculated ponies

<table>
<thead>
<tr>
<th>Virus serial passage</th>
<th>Recipient pony no.</th>
<th>Number of febrile episodes</th>
<th>Isolation success/isolation attempts</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>47</td>
<td>2</td>
<td>2/2 3/3</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>82</td>
<td>6</td>
<td>6/6 5/6</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>86</td>
<td>3</td>
<td>3/3 1/3</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>104</td>
<td>3</td>
<td>3/3 2/3</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>1</td>
<td>0/1 0/1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>101</td>
<td>2</td>
<td>0/2 0/2</td>
<td>0</td>
</tr>
<tr>
<td>2nd</td>
<td>91</td>
<td>6</td>
<td>6/6 3/6</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>5</td>
<td>5/5 0/2</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>88</td>
<td>7</td>
<td>7/7 0/1</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>4</td>
<td>1/4 1/4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>3</td>
<td>3/3 1/2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>127</td>
<td>5</td>
<td>5/5 3/4</td>
<td>8</td>
</tr>
<tr>
<td>3rd</td>
<td>53</td>
<td>6</td>
<td>6/6 1/3</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>64</td>
<td>7</td>
<td>7/7 2/3</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>117</td>
<td>1</td>
<td>1/1 0/0</td>
<td>1</td>
</tr>
<tr>
<td>4th</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5th</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6th</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>61</td>
<td>55/61 22/43</td>
<td>90% 51%</td>
<td>77</td>
</tr>
</tbody>
</table>
TABLE 5. Effect of dexamethasone treatment on ponies infected with EIAV

<table>
<thead>
<tr>
<th>Pony</th>
<th>Passage No.</th>
<th>Days after primary infection</th>
<th>No. of febrile episodes before treatment</th>
<th>Days after last febrile episode</th>
<th>Response to treatment</th>
<th>Duration of febrile episode (in days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>47</td>
<td>1st</td>
<td>200</td>
<td>0</td>
<td>0</td>
<td>negative</td>
<td></td>
</tr>
<tr>
<td>86</td>
<td>2nd</td>
<td>319</td>
<td>2</td>
<td>151</td>
<td>fever</td>
<td>1</td>
</tr>
<tr>
<td>104</td>
<td>2nd</td>
<td>180</td>
<td>0</td>
<td>0</td>
<td>fever</td>
<td>1</td>
</tr>
<tr>
<td>101</td>
<td>2nd</td>
<td>180</td>
<td>0</td>
<td>0</td>
<td>fever</td>
<td>2</td>
</tr>
<tr>
<td>86*</td>
<td>2nd</td>
<td>550</td>
<td>3</td>
<td>228</td>
<td>negative</td>
<td></td>
</tr>
<tr>
<td>96</td>
<td>3rd</td>
<td>410</td>
<td>4</td>
<td>165</td>
<td>negative</td>
<td></td>
</tr>
<tr>
<td>(Control 95)</td>
<td>2nd</td>
<td>180</td>
<td>0</td>
<td>0</td>
<td>negative</td>
<td></td>
</tr>
</tbody>
</table>

*2nd dexamethasone treatment.
<table>
<thead>
<tr>
<th>Virus serial passage</th>
<th>Recipient pony no.</th>
<th>Serial no. of febrile episodes</th>
<th>Hemoglobin (g/dl)</th>
<th>PCV (%)</th>
<th>Blood platelets (x 10^3/ul)</th>
<th>WBC (x 10^3/ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2nd 82</td>
<td>6th 11.7</td>
<td>31</td>
<td>0.52</td>
<td>11.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd 91</td>
<td>4th 14.4</td>
<td>37</td>
<td>0.90</td>
<td>10.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5th 12.0</td>
<td>33</td>
<td>1.16</td>
<td>10.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6th 7.4</td>
<td>19</td>
<td>0.54</td>
<td>4.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd 90</td>
<td>2nd 8.9</td>
<td>22</td>
<td>0.95</td>
<td>10.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3rd 8.4</td>
<td>23</td>
<td>0.98</td>
<td>6.7</td>
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</tr>
<tr>
<td></td>
<td>4th 8.9</td>
<td>24</td>
<td>0.80</td>
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</tr>
<tr>
<td></td>
<td>5th 5.7</td>
<td>15</td>
<td>0.07</td>
<td>3.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd 88</td>
<td>5th 6.5</td>
<td>18</td>
<td>1.40</td>
<td>7.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6th 5.9</td>
<td>17</td>
<td>1.40</td>
<td>10.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5th 64</td>
<td>2nd 13.8</td>
<td>36</td>
<td>0.44</td>
<td>5.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3rd 11.4</td>
<td>29</td>
<td>N.D.*</td>
<td>6.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4th 10.7</td>
<td>28</td>
<td>0.82</td>
<td>8.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6th 117</td>
<td>1st 10.2</td>
<td>28</td>
<td>3.4</td>
<td>7.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mean values in clinically healthy ponies (n = 7 ponies) 13.4 to 16 38.5 to 42 2.6 to 3.3 8.0 to 13.6

*N.D. = not done.*
<table>
<thead>
<tr>
<th>Type of EIA Disease</th>
<th>Clinical characterization</th>
<th>Pony No. and backpassage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Acute</td>
<td>short incubation period, severe clinical signs, short course</td>
<td>117 (6th)</td>
</tr>
<tr>
<td>2. Subacute</td>
<td>several febrile relapses, short course, fatal ending</td>
<td>47 (1st), 82 (2nd), 90, 91, 88, 127, 120 (3rd), 53 (4th), 64 (5th)</td>
</tr>
<tr>
<td>3. Chronic</td>
<td>frequent febrile relapses, no apparent EIA clinical signs</td>
<td>96 (3rd)</td>
</tr>
<tr>
<td>4. Inapparent</td>
<td>sporadic febrile relapses, no apparent EIA clinical signs</td>
<td>86, 104, 101, 95 (2nd)</td>
</tr>
</tbody>
</table>
TABLE 8. Effect of dexamethasone treatment on total white blood cell and lymphocyte counts in 3 ponies chronically infected with EIA virus (86, 101, and 104)

<table>
<thead>
<tr>
<th>Pony no.</th>
<th>Pre-treatment*</th>
<th></th>
<th>Second count*</th>
<th></th>
<th>Third count*</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WBC **</td>
<td>Lymphocytes **</td>
<td>%†</td>
<td>WBC</td>
<td>Lymphocytes</td>
<td>%</td>
</tr>
<tr>
<td>86</td>
<td>10.4</td>
<td>4.1</td>
<td>39</td>
<td>7.4</td>
<td>2.4</td>
<td>33</td>
</tr>
<tr>
<td>101</td>
<td>9.0</td>
<td>5.5</td>
<td>61</td>
<td>9.6</td>
<td>4.2</td>
<td>44</td>
</tr>
<tr>
<td>104§</td>
<td>10.9</td>
<td>6.6</td>
<td>61</td>
<td>11.5</td>
<td>6.9</td>
<td>60</td>
</tr>
<tr>
<td>95§</td>
<td>10.7</td>
<td>6.1</td>
<td>57</td>
<td>12.2</td>
<td>5.1</td>
<td>42</td>
</tr>
<tr>
<td>$\bar{x}$</td>
<td>10.2</td>
<td>5.0</td>
<td>54.5</td>
<td>10.2</td>
<td>4.6</td>
<td>44.8</td>
</tr>
<tr>
<td>SD</td>
<td>0.85</td>
<td>1.6</td>
<td>10.5</td>
<td>2.1</td>
<td>1.9</td>
<td>11.2</td>
</tr>
<tr>
<td>Normal values</td>
<td>5.5 - 12.5</td>
<td>2.5 - 7.5</td>
<td>55.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Blood sample collected prior to first dexamethasone administration and five and ten days after the first dose.

** x 10³/μl.

†Lymphocytes.

§Control.
TABLE 9. Effect of dexamethasone administration on lymphocyte and T and B-cell counts in ponies chronically infected with EIAV

<table>
<thead>
<tr>
<th>Pony no.</th>
<th>Lymphocytes (after separation)*</th>
<th>T-cells (%)</th>
<th>B-cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-treat** 2nd** 3rd**</td>
<td>Pre-treat</td>
<td>2nd 3rd</td>
</tr>
<tr>
<td>86</td>
<td>10 4.8 10.2</td>
<td>71.0 58.7 62.4</td>
<td>16 12.6 17.5</td>
</tr>
<tr>
<td>101</td>
<td>8.0 5.3 6.7</td>
<td>74.3 61.7 72.3</td>
<td>14 10.1 14.5</td>
</tr>
<tr>
<td>104</td>
<td>8.5 8.3 6.0</td>
<td>72.0 70.2 72.0</td>
<td>16 15.3 9.7</td>
</tr>
<tr>
<td>95+</td>
<td>7.3 10 10.3</td>
<td>73.0 72.0 75.0</td>
<td>15 15.4 20.1</td>
</tr>
<tr>
<td>X</td>
<td>8.45 7.1 8.3</td>
<td>72.5 65.6 70.4</td>
<td>15.2 13.3 15.4</td>
</tr>
<tr>
<td>SD</td>
<td>1.1 2.5 2.3</td>
<td>1.4 6.4 5.5</td>
<td>0.9 2.5 4.5</td>
</tr>
</tbody>
</table>

* x 10^3/µl of whole blood.

** Blood sample collected before first dexamethasone administration and five and ten days after the first dose.

+ Control.
TABLE 10. Stimulation index of lymphocytes from ponies 101, 104, and 86, which were chronically infected with EIAV and treated with the immunosuppressant dexamethasone

<table>
<thead>
<tr>
<th>Mitogen</th>
<th>Dose*</th>
<th>Pony number</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>101</td>
<td>104</td>
<td>86</td>
<td>95**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>SI₁⁺</td>
<td>SI₂</td>
<td>SI₃</td>
<td>SI₁</td>
<td>SI₂</td>
<td>SI₃</td>
<td>SI₁</td>
</tr>
<tr>
<td>PHA</td>
<td>100</td>
<td>87</td>
<td>29</td>
<td>113</td>
<td>65</td>
<td>33</td>
<td>64</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>53</td>
<td>37</td>
<td>97</td>
<td>46</td>
<td>35</td>
<td>49</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>26</td>
<td>30</td>
<td>47</td>
<td>20</td>
<td>13</td>
<td>24</td>
<td>5</td>
</tr>
<tr>
<td>Con A</td>
<td>100</td>
<td>44</td>
<td>51</td>
<td>49</td>
<td>19</td>
<td>31</td>
<td>31</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>10</td>
<td>17</td>
<td>15</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>PWM</td>
<td>6</td>
<td>30</td>
<td>56</td>
<td>50</td>
<td>32</td>
<td>51</td>
<td>33</td>
<td>21</td>
</tr>
</tbody>
</table>

*Micrograms.

**Control.

+Samples collected prior to (SI₁⁺), 5 (SI₂), and 10 (SI₃) days after first injection.
TABLE 11. Serum neutralization indexes of three sera collected from pony No. 82 at different times after isolation of virus P82-1 (first peak of fever).

<table>
<thead>
<tr>
<th>Virus isolate</th>
<th>Day PI</th>
<th>Serum collected on PID*</th>
</tr>
</thead>
<tbody>
<tr>
<td>P82-1</td>
<td>106</td>
<td>1.0**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.0</td>
</tr>
</tbody>
</table>

* Post-inoculation day.

** $\log_{10}$ Serum-neutralization index (positive if $\geq 2.0$).
TABLE 12. Serum-neutralization indexes of three sera collected from pony 127 at different times after inoculation. Each serum was reacted with four sequential virus isolates from the same pony.

<table>
<thead>
<tr>
<th>Virus isolate</th>
<th>Day PI</th>
<th>Serum collected on PID*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>31</td>
</tr>
<tr>
<td>P127-1</td>
<td>13</td>
<td>1.5**</td>
</tr>
<tr>
<td>P127-2</td>
<td>41</td>
<td>0.5</td>
</tr>
<tr>
<td>P127/-3</td>
<td>99</td>
<td>1.5</td>
</tr>
<tr>
<td>P127-4</td>
<td>137</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* Post-inoculation day.

** Log_{10} serum-neutralization index (positive if ≥ 2.0).
FIGURE 1. Daily rectal temperature (°C) of ponies 47 and 80 after IV inoculation of $10^6$ TCID$_{50}$/ml of an equine dermal cell adapted strain of EIAV. V = EIAV isolated from plasma samples cultured in FEK cells. + = first AGID test positive. Bars = indicate the plasma infectivity ($\log_{10}$ TCID$_{50}$/0.5 ml). DM = dexamethasone administration, first dose of a series of five injections.
FIGURE 2. Daily rectal temperature (°C) of ponies utilized as the second passage for the equine dermal-cell adapted strain of EIAV. V = EIAV isolated from plasma samples cultured in FEK cells. V = EIAV was not isolated. + = first AGID test positive. The bars indicate the plasma infectivity (log_{10} TCID₅₀ of EIAV/0.5 ml). DM = dexamethasone administration, first dose of a series of five injections.
FIGURE 3. Daily rectal temperature (°C) of ponies used as the third passage of a cell-adapted strain of EIAV. V = EIAV isolated from plasma samples cultured in FEK cells. V = EIAV was not isolated. + = first AGID test positive. The bars indicate the plasma infectivity ($\log_{10} \text{TCID}_{50}$ of EIAV/0.5 ml).
FIGURE 4. Daily rectal temperature (°C) of ponies utilized as the fourth, fifth, and sixth passage of a cell-adapted strain of EIAV. V = EIAV was isolated from plasma samples cultured in FEK cells. V = EIAV was not isolated. + = first AGID test positive. The bars indicate the plasma infectivity ($\log_{10}^{\text{TCID}_{50}}$ of EIAV/0.5 ml).
CHAPTER II
Equine Infectious Anemia:
Attempts to Titrate the Virus by Three Different Methods
INTRODUCTION

Equine infectious anemia (EIA) is an infectious disease of solipeds, also known as "swamp fever" as the disease occurs in animals raised in low lying areas.\(^1\) The etiological agent of EIA is a virus from the Family Retroviridae which resembles visna, maedi, and progressive pneumonia viruses. The virus is spherical, enveloped, and the virion has a diameter of 80 to 100 nm. The icosahedral capsid contains a heavy molecular weight RNA genome (60 to 70 S) composed of subunits.\(^2\) The virus density is between 1.16 and 1.18 g/cm\(^3\) in sucrose gradients,\(^3\) and like prototype retroviruses matures by budding from cytoplasmic membranes.\(^4,5\)

Horse leukocyte culture was the first cell system successfully used for the propagation and passage of EIAV,\(^6\) and also for its titration as the virus produces a sort of cytopathogenic effect (CPE). In addition, virulent strains of EIAV have been grown without the induction of CPE in fetal equine kidney (FEK) cells,\(^7,8\) and in an equine dermal cell line (ATCC-CCL),\(^9\) Likewise, EIAV has been grown in cells from animals of different species, including chick embryo, Hela cells, BHK\(_{21}\) cells,\(^7\) as well as human fetal lung fibroblasts.\(^10\)

Different systems have been utilized to titrate EIAV, but no one is easy and accurate. In fact, as already mentioned, EIAV has been titrated in horse leukocyte culture using CPE as an indicator,\(^11,12\) but as horse leukocytes show spontaneous degeneration it is necessary to titrate the virus by the second or the third step passage method, which makes the work difficult and tedious to perform; in addition, the system is not satisfactory because it is very hard to find a suitable donor for
the leukocytes, and an acceptable bovine serum to be used in the culture medium. A simpler technique to titrate the cell adapted strain of EIA virus in the equine dermal (ED) cell line has been described, but the results are reported as variable; therefore, the procedure is not completely satisfactory either. Likewise, EIA virus has been titrated in vivo, by injecting the infected material (blood, serum, plasma) into susceptible horses. This procedure although accurate and simple is time consuming and costly, which reduce its usefulness for EIA virus quantitation.

Murine leukemia viruses replicate in tissue culture, but do not produce CPE; consequently, serologic or bioassay methods have been required to assay their growth, and plaque type assays had not been feasible until 1970 when an indirect method of producing CPE was described.\textsuperscript{15,16} The method so called "mixed culture cytopathogenicity reaction," is based in the occurrence of syncytium formation when the XC cell line, a rat tumor cell originally induced by the Prague strain of Rous sarcoma virus is placed in contact with cells infected with murine leukemia virus; accordingly, murine leukemia virus is seeded in mouse embryo cultures from 15 to 17 day old NIH Swiss and BALB/c, and on the 5th or 6th day after infection the culture fluid is removed, and the cells are exposed for 20 to 30 seconds to ultraviolet (UV) irradiation from two GE germicidal bulbs located at 27.6 cms (60 erg/mm\textsuperscript{2}/sec). Maintenance medium containing $10^6$ XC cells is then added, the fluid is changed at 1 or 2 day intervals, the cultures are fixed with methanol and stained with hematoxylin on the 3rd or 4th day after the addition of the XC cells, and plaques are read under low magnification of a
dissecting microscope. The UV treated cultures show CPE represented by rounding and detachment of the mouse embryo cells; while the XC cells grow into a uniform monolayer. The plaques are seen as holes in the cell sheet which form as XC cells form syncytia over the infected mouse cells.

The procedure described has been used to quantitate several laboratory strains of murine leukemia virus (Gross, Moloney, Friend, Rauscher, WM-1-B, Graffi, Kirsten, and Kaplan), all tissue culture isolates of field strains tested, and naturally occurring virus in tissues of AKR, BALB/c, C3H/Bi, and C58 mice. Besides, the technique has been used to titrate replication-defective ecotropic murine leukemia viruses, by a procedure known as "helper dependent XC plaque formation."!

The syncytia infectivity assay (SIA) has been used to titrate bovine leukemia virus, as it is capable of inducing rapid syncytia formation in monolayer cell cultures of nontransformed cells from various species. In this assay bovine embryonic spleen cells, as well as F-81 cells, have been used as indicators, and the monolayer is treated with diethylamino-ethyl-dextran (DEAE-d) prior to virus inoculation; then after a 48 hour incubation, the monolayer is fixed and stained, and the results are given in syncytia forming units (SFU).

The inhibition of viral replication by the presence of other viruses (interference) has been used to titrate some non-cytopathogenic viruses; for instance, hog cholera and Sindbis viruses (Togaviridae) induce a transient refractory state to vesicular stomatitis virus (Rhabdoviridae). This type of interference is known as heterologus
viral interference. Laude\textsuperscript{24}, and Fukusho \textit{et al.}\textsuperscript{25}, report the reverse plaque formation (RPF) by hog cholera virus inducing interference with vesicular stomatitis virus (VSV), and how this phenomenon is used as a rapid and simple procedure to quantitate several strains of hog cholera virus (HCV). In this procedure, they grew the swine kidney cell line SK-H, and swine testicle (ST) cells\textsuperscript{25}, and when the monolayer was confluent they inoculated HCV; then, after viral adsorption the monolayer was covered with a 3\% methylcellulose solution, and after 2 days of incubation at 37°C, the methylcellulose was removed and the monolayer infected with VSV at a multiplicity of infection of about 2 plaque forming units/cell; finally, after an incubation of 15 to 20 hours at 37°C, the plates were stained and the reverse plaques visualized and counted. The plaques were produced as colonies of HCV infected cells which were refractory to VSV infection, standing out in the background of a VSV-sensitive, disintegrated cell sheet. Incubation for 3 and 4 days gave plaques ranging from 1 to 3 mm, and from 2 to 5 mm in diameter respectively. Cells in a circular plaque were all intact and surrounded by disintegrated cell debris; equally important, reverse plaques could be also developed on replicated dishes under a liquid medium without methylcellulose overlay medium.

The objectives of this study was to determine whether EIAV can be titrated by the mixed culture cytopathogenicity reaction, the syncytia-infectivity assay, and/or the reverse plaque assay.
MATERIALS AND METHODS

THE MIXED CULTURE CYTOPATHOGENICITY ASSAY

**Virus** - The equine infectious anemia virus used in all the trials was an EIAV stock N°376 (Wyoming strain), with a titer of $10^{5.5}$ TCID$_{50}$ of EIAV/0.5 ml, obtained from Dr. C.J. Issel, Department of Veterinary Science, Louisiana State University.

**Cell Cultures and Mediums** - FEK cells prepared at our Department were used as substrate to grow EIA virus. The cells ($1 \times 10^5$) per well were grown in multi-well plates with 6 flat bottom wells, with 9.6 cm$^2$ approximately$^a$ (growth area). The cultures were fed with Eagles minimal essential medium$^b$ with 25 mM Hepes$^c$, 100 micrograms of streptomycin and 100 U of penicillin/ml (MEM), supplemented with 10% bovine fetal serum$^b$.

**XC Cells** - (ATCC CCL 165)$^{26}$ are a certified cell line derived in 1961 by D. Simkovic et al. from a transplantable tumor developed by J. Svoboda in 1960. The tumor was induced in newborn white-Weston rats by the intramuscular injection of the Prague strain of Rous sarcoma virus. The XC cells used to conduct this research were obtained in the passage 109, from Dr. G. Amborski from the Cell and Organ Culture Laboratory, at the School of Veterinary Medicine, Louisiana State University. The cells were maintained at 37°C and refed every other day with MEM$^b$ additioned of 3% bovine fetal serum$^b$.

**Ultraviolet Irradiation** - Equine infectious anemia virus infected and noninfected FEK cells were exposed to UV irradiation for 30, 60, 90, 120, 150, and 180 seconds, from a germicidal UV lamp, Sylvania G 30T8 located at 70 cms, which provides an intensity of irradiation of 116.6
microwatts/cm². A 60 second exposure was enough to kill the cells in 24 to 48 hours.

**Procedures** - Primary FEK cells were seeded in multiwell plates (6 wells per plate) in the amount of $1 \times 10^5$ cells/well. When about 75% of the monolayer was formed ten-fold dilutions of stock EIAV were seeded in the respective wells in 13 replications. EIAV was allowed to adsorb for 60 minutes at 37°C; then the excess virus was washed off with phosphate-buffered saline solution (PBS), pH 7.4, the monolayer refed, and the cells incubated at 37°C. Medium was changed every 3rd day and on day 12 the spent media was harvested and processed to determine the presence of EIAV antigen (p26) with an agar gel immunodiffusion test, using reagents prepared in our laboratory. If EIAV was not detected, the procedure was repeated until EIAV antigen was detected. Then, the cell monolayers were irradiated for 60 seconds, and 1 ml of a solution containing $10^6$ XC cells was added/well. The cells were fed with MEM supplemented with 10% bovine fetal serum, and every 24 hours thereafter with MEM supplemented with 3% bovine fetal serum. After four days, the medium was removed and the monolayers fixed and stained with 2 mls of a solution of 10% buffered formalin with 0.1% crystal violet for 5 minutes. The stained preparations were examined for plaques and/or syncytia formation (cells containing five or more nuclei were scored as syncytia); therefore, to minimize counting errors, ten randomly selected fields ($0.55 \text{ mm}^2$) were counted per well using an inverted microscope (Olympus) with a 10X ocular and 20X objective. The number of syncytia counted per well was recorded as number of multinucleated cells per virus dilution. A total of 30 control wells were studied which
consisted of FEK cells not infected, but treated the same as the EIAV infected FEK cells

**Test Statistics** - The students t test was used to evaluate the results obtained, to determine first, differences in the number of syncytial cells counted within each one of the EIAV dilutions; second, differences in the mean number of syncytia counted among dilutions; third, differences in the number of syncytia counted in the control cells; fourth, differences in the number of syncytia counted in the test and control cells; in addition, the linear regression and correlation techniques were applied to the Y values (mean number of syncytia counted per EIAV dilution), to test for linear relationship with X (EIAV dilutions), and to measure the relative strength of the relationship between variables (correlation).

**THE SYNCYTIA INFECTIVITY ASSAY (SIA)**

**Virus** - The same EIAV stock already described was used in the SIA.

**Cell Cultures and Mediums** - XC cells already described were used as indicator cells, and MEM*, additioned of 10% or 3% bovine fetal serum used to start and to maintain the cells

The polycation DEAE-d was utilized to enhance virus penetration into the cells.

**Procedure** - XC cells were seeded in multi-well culture plates (24 wells per plate)⁴, at a concentration of 1.5 X 10⁵ cells per well, and incubated at 37°C for 24 - 48 hours to form a layer of cells that was 75% confluent. Growth medium was removed and the cells were treated with 25 micrograms of DEAE-d per ml, using 0.5 ml per well. After 60 minutes at 37°C the DEAE-d was removed, and the cells were inoculated
with 0.1 ml of ten fold dilutions of EIAV stock, respectively, in 6 replications per virus dilution. The virus was allowed to adsorb for 60 minutes at 37°C; then the virus excess was washed off with PBS with a pH of 7.4, and the cells refed with MEM supplemented with 3% fetal calf serum. Finally, after 24 hours, the cells were washed twice with PBS, fixed and stained with a solution of 10% buffered formalin with 0.1% crystal violet. Polykaryocytes containing 5 or more nuclei were recorded as syncytia, and counted in 10 randomly selected fields per well using an inverted microscope (Olympus) with 10X ocular and 20X objective. As controls, six wells were treated with DEAE-d but received no EIAV, and six additional wells were left without any treatment (neither EIAV nor DEAE-d). The cells were fixed and stained and the polykaryocytes counted in the same way described for the test cells.

**Test Statistics** - The student t test was used to establish first, differences in the mean number of syncytia counted within each EIAV dilution; second, differences in the mean number of syncytia counted among the EIAV dilutions; third, the effect of DEAE-d treatment on EIAV infected and noninfected XC cells. Finally, the linear regression and correlation techniques were applied to the Y values (mean number of syncytia counted per EIAV dilution) to test for linear relationship with X (EIAV dilutions), and to measure the relative strength of the relationship between variables (correlation).

**THE REVERSE PLAQUE ASSAY**

**Viruses** - Equine infectious anemia virus stock No. 376 was used as in the previously described assays. In addition, a battery of viruses including VSV (New Jersey strain), equine herpes virus-1, equine
rhinovirus-1, equine adenovirus, and equine viral arteritis virus were assayed on FEK cells for their ability to induce plaque formation, and then be used as challenge virus for EIAV infected cells in an attempt to induce reverse plaque formation. Plaques were formed by equine herpes virus-1 and by vesicular stomatitis virus, but the other 3 viruses tested did not induce good plaque formation, then VSV was selected as challenge virus as it induced a better plaque formation.

**Cell Culture and Mediums** - Fibroblastic FEK cells were used as they are more susceptible to VSV, than epithelial cells. The cells were seeded and incubated in multi-well plates at $1 \times 10^5$ cells/well and incubated for about 48 hours or until the monolayer was usually complete. The cells were fed with MEM supplemented with 10% bovine fetal serum, and prepared as already described for the aforementioned assays. But after EIAV infection, the cells were fed with a 2% methylcellulose overlay in MEM supplemented with 2% fetal calf serum.

**Procedure** - Fibroblastic FEK cells ($1 \times 10^5$/well) were seeded in multi-well plates, and when the monolayer was complete it was infected with repetitive dilutions of EIA in the amount of 0.1 ml per well in 12 replications. Six wells were not infected and acted as controls. After virus was added, it was allowed to adsorb for 30 minutes at 37°C, the plates being shaken at 10 minutes interval; then, the excess virus was washed off and the monolayers overlayed with methylcellulose, and incubated for 12 to 14 days, the medium being changed every 3 days. In addition, 12 replications of the titration were made by adding liquid medium after EIAV infection, instead of overlaying methylcellulose.
After 12 to 14 days of incubation the wells were depleted of medium (the medium subsequently was assayed for EIAV p26 antigen by the AGID test), and VSV added at the multiplicity of infection of 2 to 5 plaque forming units (PFU)/cell, or in other trials 50 (PFU) per well. The virus was allowed to adsorb for 60 minutes, and then the plates were refed, and finally incubated for 15 to 20 hours at 37°C. At the end of this incubation, the monolayers were fixed and stained with 1.5 mls of a solution of 10% buffered formalin with 0.1% crystal violet per well for 5 minutes. The plates were finally examined for plaques, or for reverse plaque formation.

RESULTS

THE MIXED CULTURE CYTOPATHOGENICITY ASSAY

The assay was carried out by a procedure described to titrate murine leukemia viruses, but introducing some necessary modifications. From the observation of the XC cells it was found that mitotic figures are constantly found; in addition, many giant cells are seen with 2 to 3 nuclei. When XC cells were overlayed onto previously irradiated uninfected and infected FEK cells the XC cells completely replaced FEK cells without plaque formation, but with formation of giant syncytial cells containing up to 18 nuclei (Table 1).

From Table 1 it can be seen that the number of polykaryocytes counted was variable within each EIAV dilution, and at each replication; accordingly, the statistical analysis indicated the following: (i) there was no difference in the number of syncytia counted in the EIAV dilutions $10^{-1}$ to $10^{-3}$, but there was significant difference in the number of polykaryocytes counted in the dilution $10^{-4}$ to $10^{-6}$ ($P \leq 0.05$);
nevertheless, the counts were variable from one replication to another; (ii) there was not difference in the mean number of syncytia counted among the EIAV dilutions (P≤0.05); (iii) there was a significant difference between the number of syncytia counted in the EIAV infected and noninfected (control), FEK cells (P≤0.05); (iv) there was no true regression in the number of syncytia formed per EIAV dilution (see Figure 1). These results indicate that, the mixed culture cytopathogenicity assay is not useful to quantitate EIAV, in a similar manner as it has been used to titrate murine leukemia viruses.

THE SYNCYTIA INFECTIVITY ASSAY

Attempts were made to infect XC cells with EIAV without success. Table 2 shows the number of polykaryocytes counted per EIAV dilution in 6 replications. The data from Table 2 indicate that the number of syncytia counted did not vary within each EIAV dilution; as a result, the statistical analysis yielded the following results: (i) there was no significant difference in the number of syncytia counted within each one of the EIAV dilutions (P≤0.05); (ii) there was no significant difference in the number of polykaryocytes counted among the EIAV dilutions (P≤0.05); (iii) there was no true regression in the number of syncytia formed per EIAV dilution (see Figure 2); (iv) no syncytia were found in the control cells. Therefore, these results suggest that the SIA is not suitable for the quantitation of EIAV.

THE REVERSE PLAQUE ASSAY

Vesicular stomatitis virus was overlayed onto EIAV infected FEK cells, at the multiplicity of infection of 2 to 5 PFU/cell. It was observed that in the monolayers overlayed with methylcellulose, the
cells remained intact after infection giving a false impression of true heterologus interference between EIAV and VSV; on the contrary, the cells fed with liquid medium showed a large number of plaques, visible under the microscope only, or after staining 15 to 20 hours after VSV challenge. On the other hand, when 50 PFU of VSV per well were used, more discrete plaques were observed in the monolayers fed with liquid medium, but the monolayer was completely destroyed 36 hours after VSV infection. No difference was found with the control cells, which were also completely destroyed by VSV, and there was no reverse plaque formation. Consequently, these results indicate that EIAV does not interfere with the superinfection of FEK cells by VSV, and that no reverse plaques are formed in FEK cells monolayers previously infected with EIAV, and then challenged with VSV; therefore, the reverse plaque assay seems to be not suitable for the quantitation of EIAV using VSV as challenge virus.

DISCUSSION
THE MIXED CULTURE CYTOPATHOGENICITY ASSAY
When XC cells are overlayed onto non murine leukemia virus (FLV) infected mouse embryo cells, the XC cells invade and completely replace the monolayer; whereas, when overlayed onto FLV infected mouse embryo cells, countable plaques are formed which is advantageously used to titrate murine leukemia viruses.\textsuperscript{15,16} In our case, when XC cells were overlayed onto a complete monolayer of non-irradiated EIAV infected and noninfected cells, the XC cells did not grow probably due to lack of an attachment surface. On the contrary, when XC were overlayed onto previously irradiated EIAV infected and noninfected FEK cells, the XC
cells completely replaced the FEK cells in 72 hours and showed syncytia formation within hours of contact (syncytia from without).

The formation of syncytia is due to cell fusion, which can occur independently of virus replication and can be induced by infective and inactivated virus (cell fusion from without), or occurs in the later stages of virus replicative cycle, following infection of cells with moderate or low doses of virus (fusion from within). However, the exact mechanism of cell fusion is unknown, although much effort has been put into its elucidation during the past decade. Howe and Morgan have shown that the envelope of Sendai virus is able to fuse with the plasma membrane of erythrocytes; therefore, it is conceivable that an enveloped virus particle in close association with the membrane of two different cells, fuses with each permitting the formation of a cytoplasmic bridge between the two. This hypothesis would explain the fusion from without and within on the same bases, i.e., bridge formation between the virus glycoproteins (gp90 and gp45 in EIAV) and normal cell membranes.

Equine infectious anemia virus does not appear to replicate on XC cells, as we failed to detect the antigen in the cell culture supernatants, even by trying to alter the XC cell membrane permeability with DEAE-d; nevertheless, we found that more syncytia were formed at the lower EIAV dilutions (10^{-1} to 10^{-3}). This finding suggests that the syncytia formation would be mediated by EIAV external glycoproteins, that the cell fusion occurring would be due to cell fusion from without, and that syncytia formation is enhanced by placing XC cells in contact with virus producing cells. Finally, although syncytia formation seems to be induced by contact of EIAV and XC cells, the mixed culture
cytopathogenicity assay is not adequate to titrate EIAV, because the number of syncytia counted varied very much within each EIAV dilution and with each replication and there was not true regression between the mean number of syncytia counted and the virus dilutions. It is also worthy of mention, that it is very difficult to maintain healthy FEK cells in reduced growth areas, and that the need persists to find an accurate and easy method to quantitate EIAV.

THE SYNCYTIA INFECTIVITY ASSAY (SIA)

Bovine leukemia virus (BLV), a type C leukemia virus is able to induce rapid syncytia formation in monolayer cell cultures of nontransformed cells from various species, a phenomenon that is successfully used to quantitate the virus in a syncytia infectivity assay.\textsuperscript{20,21} A similar technique was used in this study to try to quantitate EIAV, but only a low number of syncytia were formed per EIAV dilution, while in the SIA for BLV syncytia are formed in large amounts, therefore allowing BLV titration, as the syncytia formation decreases as BLV dilutions increase, giving a correlation between syncytium formation and the amount of BLV.\textsuperscript{20}

As it was previously mentioned, XC cells appear to not support the growth of EIAV; thus, the syncytia formed after EIAV antigens and XC cells membrane contact would be due to a cell membrane fusion from without, as it occurs in the SIA for BLV.\textsuperscript{20,21} On the other hand, the treatment of the indicator cells (XC cells) with DEAE-d would not enhance the EIAV cell attachment and penetration; hence, the syncytia observed in the indicator cells would be produced by the fusion of two or more cell membranes by the outermost EIAV proteins (gp90 and gp45),
or as a result of cell membrane injuries (by the viral antigens) which join together to compensate for such injuries (cell membrane dissolution, etc).\textsuperscript{27} As a result of this study, the titration of EIAV cannot be obtained by the SIA, at least under the conditions of this study; therefore, other research efforts should be considered in the search of an easy and reliable system to titrate EIAV.

\textbf{THE REVERSE PLAQUE ASSAY}

Cells infected by more than one virus may support the normal replication of them, or one virus may inhibit or enhance the multiplication of other. Virus induced inhibition of viral replication is called "the interference phenomenon."\textsuperscript{24} Such phenomenon has been advantageously utilized to titrate hog cholera virus (HCV), which is noncytopathogenic, and the procedure has been known as "the reverse plaque assay."\textsuperscript{11,23,25} The method is based on the complete resistance of the HCV infected cells to superinfection by VSV. In this study, the aim was to determine whether the system could be used for EIAV titration. The results showed that when EIAV infected FEK cells which had been overlayed with methylcellulose were challenged with VSV, CPE did not occur giving the impression that EIAV actually interfered with cells superinfection with VSV; however, as the assay was also carried out using liquid medium (instead of methylcellulose), the monolayers showed extensive CPE 15 to 20 hours after VSV superinfection, and were completely destroyed after 36 hours. This finding indicated that EIAV infection does not interfere with superinfection of FEK cells with VSV. It was also concluded that VSV did not reach the cells (the cells fed with the methylcellulose overlay) because a thin film of the semisolid...
medium covered the monolayers. This film was not removed despite several washes with PBS. In the reverse plaque assay as reported by Fukusho et al.\textsuperscript{25} and Laude\textsuperscript{23}, the reverse plaques were clearly visible as intact cell colonies in the background of a disintegrated cell sheet, and the relationship between virus concentration and the number of plaques was essentially linear.\textsuperscript{25}

It should be finally mentioned that 12 to 14 days were required for EIA virus to grow before VSV challenge, and as already stated, it was very difficult to keep FEK cells in good shape in a reduced growth area; on the contrary, in the reverse plaque assay used to titrate HCV only 2 or 4 days of virus incubation are required before VSV challenge.\textsuperscript{23,25}

Consequently, as EIAV does not protect FEK cells against superinfection with VSV (no reverse plaques are formed), the system as carried out in this study is not suitable for EIAV quantitation.

**SUMMARY**

A study was conducted to determine whether the mixed culture cytopathogenicity assay (XC cell assay), the syncytia infectivity assay (SIA), and the reverse plaque assay (RPA) would be suitable to quantitate equine infectious anemia virus.

The XC cell assay has been used to titrate several strains of murine leukemia virus, which do not induce cytopathogenic effect in tissue culture, but indirect syncytia and plaque formation occur in XC cells, when placed in contact with cells infected with murine leukemia virus. The SIA on the other hand, is utilized to quantitate bovine leukemia virus, as it is capable of inducing rapid syncytia formation in monolayer cell cultures of nontransformed cells from various species.
Finally, the RPA has been used to titrate some noncytopathogenic viruses, like hog cholera virus and Sindbis virus, which induce a transient refractory state of the cell to superinfection with vesicular stomatitis virus. Challenge of cells previously infected with hog cholera virus, results in the formation of islands of cells (reverse plaques) surrounded by disintegrated cell debris.

The results of this study indicate that none of these techniques is accurate to quantitate EIAV, under the conditions utilized to carry out the assays.
REFERENCES


FOOTNOTES

b. Gibco Laboratories, Grand Island, NY.
c. Sigma Chemical Company, St. Louis, MO.
TABLE 1. Number of syncytia counted per EIAV dilution in the XC cell assay

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<th>Replications</th>
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\[ \bar{X} = 5.7 \quad 3.6 \quad 3.4 \quad 3.2 \quad 2.7 \quad 2.3 \]

\[ SD = 4.2 \quad 3.0 \quad 2.3 \quad 1.6 \quad 2.1 \quad 2.1 \]
TABLE 2. Number of syncytia counted per EIAV dilution in the syncytia infectivity assay

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**X**

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FIGURE 1. Mean number of syncytia counted per EIAV dilution in the mixed culture cytopathogenicity assay and regression line ($r = \text{regression coefficient}$).

$y = 5.47 - 0.56x$

$r = 0.89$
FIGURE 2. Mean number of syncytia counted per EIAV dilution in the syncytia infectivity assay and regression line ($r = \text{regression coefficient}$).

\[ y = 0.92x + 2.2 \]

$\text{EIAV DILUTIONS (LOG 10)}$

$\text{SYNCYTIA PER VIRUS DILUTION (x)}$
VITA

Alberto Orrego Uribe was born September 8, 1942, in Manizales, Colombia, South America. He was the fifth son of Mr. Francisco Orrego and Mrs. Guillermina Uribe. He graduated from Nuestra Senora High School in 1960, and in 1961 entered the University of Caldas. The degree of Doctor of Veterinary Medicine was awarded to him in December, 1966. Following graduation, he joined the Instituto Colombiano Agropecuario where he served as field and diagnostic veterinarian until 1972 when he joined the School of Veterinary Medicine at the University of California (Davis). The degree of Master of Preventive Veterinary Medicine was awarded to him in August, 1974. He married Ms. Ofelia Gomez in 1971 and had three daughters, Ana Cristina, Beatriz Eugenia, and Constanza Maria.

He entered the Graduate School of Louisiana State University in January, 1980 to conduct research funded by the Agricultural Center. His major field of study was Veterinary Medical Sciences with a minor in epidemiology. The research that he conducted was to generate equine infectious anemia virus candidates, from which the antigenic variation of the virus might be elucidated.
EXAMINATION AND THESIS REPORT

Candidate:  Alberto Orrego Uribe

Major Field:  Veterinary Medical Sciences

Title of Thesis:  Equine Infectious Anemia Virus: (1) the generation of Virus variant candidates; (2) attempts to titrate the virus by three different methods.

Approved:

[Signature]
Major Professor and Chairman

[Signature]
Dean of the Graduate School

EXAMINING COMMITTEE:

[Signature]

[Signature]

[Signature]

[Signature]

Date of Examination:

May 11, 1983