1987

Characterization of Equine Infectious Anemia Virus Variants Generated in Vivo and in Vitro and a Rapid Assay for Virus-Specific Antibody.

Paul Murumba Rwambo
Louisiana State University and Agricultural & Mechanical College

Order Number 8811435

Characterization of equine infectious anemia virus variants generated *in vivo* and *in vitro* and a rapid assay for virus-specific antibody

Rwambo, Paul Murumba, Ph.D.

The Louisiana State University and Agricultural and Mechanical Col., 1987

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark ✓.

1. Glossy photographs or pages ✓
2. Colored illustrations, paper or print _____
3. Photographs with dark background ✓
4. Illustrations are poor copy _____
5. Pages with black marks, not original copy _____
6. Print shows through as there is text on both sides of page _____
7. Indistinct, broken or small print on several pages ✓
8. Print exceeds margin requirements _____
9. Tightly bound copy with print lost in spine _____
10. Computer printout pages with indistinct print _____
11. Page(s) _____ lacking when material received, and not available from school or author.
12. Page(s) _____ seem to be missing in numbering only as text follows.
13. Two pages numbered _____. Text follows.
14. Curling and wrinkled pages _____
15. Dissertation contains pages with print at a slant, filmed as received ✓
16. Other _____

_____

_____

CHARACTERIZATION OF EQUINE INFECTIOUS ANEMIA VIRUS VARIANTS GENERATED IN VIVO AND IN VITRO AND A RAPID ASSAY FOR VIRUS-SPECIFIC ANTIBODY.

A Dissertation

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

in

Interdepartmental Program

In

Veterinary Medical Sciences

by

Paul Murumba Rwambo
B.V.M., University of Nairobi, 1980
December 1987

ACKNOWLEDGEMENTS

Sincere thanks are to my major professor Dr. Charles J. Issel, Professor of Veterinary Microbiology and Parasitology, for his support throughout the duration of my program. Special thanks are also to him for his honest criticisms, technical and financial support that allowed me to accomplish my academic pursuits.

Much thanks are also due to members of my graduate committee Drs. Ronald C. Montelaro, Kenneth L. Schnorr, Grace F. Amborski and William J. Todd for their invaluable support along the course of my training.

Enormous thanks are also due to Dr. Storz, Head of the Department of Veterinary Microbiology and Parasitology, for his professional and financial support.

Sincere thanks are due to members of the Dept. of Veterinary Science especially Mr. W. V. Adams Jr., Ms Jackie McManus and Ms Sue Hagius for their technical support. Much thanks are also to Dr. Vida Dennis for her technical support.

Very sincere thanks are to my parents, Virginia Njeri Rwambo and Godfrey Rwambo, my wife, Mrs. Lucy W. Murumba, and our children Rwambo, Njeri and Nyaga, for their patience, love and moral support throughout the duration of my training. I sincerely owe a lot to **"y'all".**

TABLE OF CONTENTS.

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Three Shetland ponies (A, B and C) were inoculated with plasma from a pony with clinical signs of equine infectious anemia (EIA) and observed for 165 to 440 days. Clinical signs of EIA marked by high fever (39.5^0C) lasting 2-4 days were observed 2-3 weeks after infection and one to four such episodes occurred in each pony. EIAV was reisolated from plasma by end-point dilution in fetal equine kidney (FEK) cells and a plasma viremia $10^{3.5}$ $TCID_{50}$/ml was observed during each episode. A total of seven virus isolates were recovered.

Cross-neutralization tests with sequential serum samples showed that the four viruses isolated from pony A during febrile episodes 4-5 weeks apart were serologically different. Tryptic peptide map analysis of these isolates revealed additions or deletions between each isolate and its immediate predecessor. Western blot analysis of these four isolates including those from ponies B and C with a panel of monoclonal antibodies confirmed antigenic variation in both gp90 and gp45. Both conserved and variable epitopes were identified in each glycoprotein with greatest variation in gp90. Analysis with MAbs confirmed similar antigenic variation in an in vitro variant generated in cell culture.

The humoral response to EIAV was monitored for anti-virus antibodies by AGID, Western blots and a novel single-step immunoblot devised in this study for rapid assay of EIAV antibodies and antigens. Specific IgG directed against determinants of EIAV gp90, gp45 and p26 in homologous and heterologous virus isolates was detectable within one month after infection. The group-specific determinants in gp90 and gp45 were more antigenic than those in p26. Neutralizing antibodies first

detectable within two months were observed to be effective only to viruses isolated prior to serum collection but became broadly reactive later in the infection regardless of the number of clinical episodes.

Our data showed that rapid antigenic variation occurred during persistent infection with EIAV in ponies and in cell culture in the presence of immune serum, and neutralizing antibody response broadened during the course of EIA. The conserved antigenic determinants of EIAV gp90 and gp45 identified in this and previous studies will be useful in diagnostic procedures and may have potential for vaccines against EIA.

CHAPTER ONE

LITERATURE REVIEW

EQUINE INFECTIOUS ANEMIA (EIA): A LENTIVIRUS DISEASE OF HORSES.

Equine infectious anemia (EIA) is a naturally occurring viral disease of all Equidae. The disease has a world-wide distribution and is characterized by recurrent episodes of fever, hemolytic anemia, bone marrow depression, lymphoproliferation, a life long persistent infection and immunologically-mediated lesions (18, 29, 36, 68, 107). The etiological agent of EIA is an exogenous replication competent retrovirus that is more closely related to the lentiviruses than to the other subfamilies of retroviruses (15, 16, 56, 125, 133, 165). The disease has a variable clinical course; but in natural infections, the disease is mostly inapparent (77). The nature of EIA virus (EIAV) induced clinical signs, the presence of immunologically mediated lesions and the relatedness of this equine virus to both human and other animal lentiviruses make EIA a good model for the elucidation of mechanisms involved in the persistence of viral infections in immunologically responding hosts. In addition, it may be useful for the evaluation of various chemotherapeutic and immunoprophylactic protocols for the control of rapidly evolving viruses. Thus, an evaluation of the mechanisms involved in the persistence of EIAV in susceptible horses is paramount in the eventual design of effective disease control protocols. Foremost amongst these is understanding how horses respond immunologically to EIAV, and how

antigenic drift may be responsible for the recurrence of febrile episodes in chronic EIA. The detailed serological and biochemical analyses of viruses isolated from horses with either acute or chronic EIA may aid in estimating the degree of antigenic and genetic variation in EIAV.

Equine infectious anemia has a variable clinical course. Horses that are infected with equine infectious anemia virus (EIAV) display a spectrum of disease conditions that range from an inapparent state to a chronic infection that is accompanied by recurrent disease episodes. Following primary exposure to EIAV, horses may remain inapparent for long periods or have an acute disease that may result in death or recovery. Horses that survive an acute episode may in turn become inapparent carriers or progress to a chronic infection. In natural cases of EIA a great majority of the horses are inapparent carriers of the virus and are only discovered when their serum tests positive for anti-virus antibody (77).

Acute EIA is most often associated with the first exposure to the virus. The incubation period varies from 5 days to as long as several months depending upon the virus isolate used, the amount of virus given, the host resistance and environmental factors (82). Pyrexia ($>39.5^0$ C), which lasts for 3 or 4 days, is the most characteristic clinical sign and is generally evident from 7 to 30 days after infection. Horses with acute EIA are often not diagnosed since precipitating antibodies detectable in the agar gel immunodiffusion (AGID) test peak at about 3 to 4 weeks after infection (29). During the peak of the febrile response, high viremia is often found and viral antigen can be demonstrated in many body organs, particularly in those rich in macrophages (91, 106). The

virus is thought to cause a lytic infection of macrophages (87, 106). Most horses with acute EIA survive and may go unnoticed as the classical signs of the disease, i.e. weight loss, anemia and ventral edema, are usually not evident during this first episode (77).

Following the initial infection, chronic EIA may develop. It is characterized by the occurrence of unpredictable recurrent clinical episodes that may be several weeks to months apart (91). The number and the frequency of such episodes is variable but tends to decline with time and about 90% occur within one year of infection (91, 122). Horses with chronic EIA are positive in AGID test and depict most of the signs associated with EIA including fever, loss of weight, weakness, anemia, ventral edema and terminal depression (122).

The level of cell-free viremia in horses with chronic EIA is variable but is highest during the recurrent febrile episodes when it reaches 10^4/ml of plasma for 2 to 3 days before subsiding (91). Horses with chronic EIA are apparently normal between febrile episodes; isolation of virus from plasma during afebrile periods is difficult and may be indicative of low levels of circulating cell-free virus. The repeated massive outpouring of virus and associated antigens in the face of a vigorous immune response sets in motion a series of immunopathological events that cause the clinical signs and lesions of EIA and include anemia, infectious immune complexes and glomerulonephritis (67, 107, 139). Mares that become infected while carrying a foal may either abort or deliver infected foals depending on the clinical course (81). After an unpredictable number of recurrent febrile episodes, most horses stop cycling and remain inapparent carriers of EIAV. However, a number of

EIAV infected horses may fail to show any signs of disease but remain inapparently infected (77). Horses that have been apparently normal for a long period of time may suddenly re-experience an acute febrile episode. Moreover, such inapparently infected horses may be induced to experience clinical EIA by exposing them to environmental stress such as hard work or by immunosuppressive therapy, such as multiple doses of dexamethasone (77, 92). These data all indicate that once horses are infected they remain virus carriers for life, irrespective of the course of the disease.

EIAV can be naturally transmitted from acutely ill horses by the interrupted feeding of horse flies (79). Since the virus has not been shown to multiply within insects, the only known mode of transmission other than in utero is mechanical (145). Mechanical transmission of EIAV can also be affected by the transfer of blood or blood products on contaminated instruments or syringes. The success of mechanical transmission of viruses depends on many variables including the level of viremia at the time of feeding (or transfer of body fluids), the survivability of the virus outside the natural host, the population density of both the natural host and the transmitting insect(s) and the seasonality and the feeding habits of the insect vector population. Within the U.S., Louisiana has the highest reported incidence of EIA (78).

Diagnosis of EIA currently depends on the detection of precipitating antibodies in the AGID test, popularly known as the Coggins test (28). The test is based on the detection of antibodies reactive to the viral antigens, particularly the major core protein, p26, which contains group specific determinants. The correlation between AGID-seropositivity and

the presence of infectious virus in the blood of test horses may approach 100% (30). Although other more sensitive serological tests including immunofluorescence (35), radioimmunoassay (32), and enzyme linked immunosorbent assay (140) have been developed, they are rarely used. Like all other serological tests based on the detection of serum antibody, the diagnosis of EIA by AGID is limited to the phase of the disease when precipitating serum antibody is present. Other diagnostic procedures that have been used in the diagnosis of EIA include hematology, pathology, and the horse inoculation test (77).

RETROVIRUSES AND THEIR CLASSIFICATION.

Retroviruses are classified according to their morphology, the structure of their RNA viral genomes (a dimer of two identical subunits of single-stranded RNA (30S-40S) of positive or messenger sense polarity), and their RNA-dependent DNA polymerase (reverse transcriptase) (47). Retroviruses are widely distributed among vertebrate species, including mammals, birds, and reptiles (154). The process of reverse transcription in the expression of genetic information is a strategy utilized not only by the retroviruses, but also by hepadnaviruses, as well as viruses infecting insects and plants (73). The family Retroviridae is divided into three subfamilies: 1) Oncovirinae, including all the oncogenic members and many closely related nononcogenic viruses; 2) Lentivirinae, the "slow" viruses, such as visna virus; and 3) Spumavirinae, the "foamy" viruses that induce persistent infections without any classical disease (154).

Lentiviruses are so named because they cause diseases with long incubation periods, insidious onsets and slowly progressive courses (61).

The prototype members of this subfamily of retroviruses include a number of viruses that have been isolated from sick sheep and goats in various locales. These viruses include visna, maedi (59), zwoegerziekte (40), progressive pneumonia virus (PPV) (83), and caprine arthritis encephalitis virus (CAEV) (34). Other lentiviruses include: equine infectious anemia virus (EIAV) of horses (15); a number of related retroviruses that have been isolated from immunodeficient (39) and asymptomatic monkeys (80); and the retroviruses associated with the acquired immunodeficiency syndrome (AIDS) in humans, the human immunodeficiency virus (HIV) (10, 23, 27, 57)

MORPHOLOGY.

Mature EIA virions are enveloped spherical particles that are 110 to 120nm in diameter (158). The external surface of the virion envelope contains glycoprotein projections (spikes and knobs) that are 10 to 12nm in length (158). The core of the virion is remarkably pleomorphic and ranges from cone shaped to tubular (158). An inner particle shell is located between the core and the envelope. Ultrastructurally EIAV closely resembles visna virus and the other members of the subfamily lentivirinae (56). Recent evidence indicates that members of the subfamily lentivirinae are morphologically related and "mature" particles contain a condensed central core, which depending on the section plane, appear eccentric, bar-shaped, triangular or oblong (13, 50, 57). Mature EIA virions acquire their envelope by budding from cytoplasmic membranes, both into cytoplasmic vacuoles and extracellularly (56).

PHYSICOCHEMICAL PROPERTIES OF EIAV.

The physicochemical properties of EIAV are those of enveloped viruses in general but those of retroviruses in particular. Mature EIA virions are enveloped and have a buoyant density of about 1.16 g/ml in cesium chloride (16, 102). Virion infectivity is sensitive to ether and detergents but is resistant to trypsin treatment at 37^{0}C, ultra-violet and X-irradiation (114). Virus infectivity is inactivated following treatment with a variety of chemical disinfectants including sodium hydroxide, sodium hypochlorite, organic phenolic compounds and chlorhexidine (144). In horse serum, EIAV has been reported to be inactivated by treatment with 56^{0}C for 60 min (9). EIAV has been mechanically transmitted to horses by groups of 25 horse flies 30 min but not 4 hours after they had taken a partial blood meal from an acutely infected donor (66).

The cell adapted Wyoming strain of EIAV (98) has been the source of virus for most the majority of published reports on EIAV since 1974. Purified EIAV has been characterized extensively. It is composed of the following: a high molecular weight RNA (60 to 70S) made of two identical linear subunits that are noncovalently bound near their 5'end; a RNA-dependent DNA polymerase (reverse transcriptase) that has a preferential requirement for Mg++; a lysine-specific tRNA molecule noncovalently bound near the 5' end of each genomic RNA and functions to prime reverse transcription and 6 major structural polypeptides analogous to those of prototype C-oncogenic retroviruses (6, 15, 16, 112, 125).

VIRAL GENOME: STRUCTURE AND EXPRESSION.

The genome of EIAV possesses the three classical genes found in all replication competent retroviruses (26). These are the gag gene which codes for the internal structural proteins, the pol gene which codes for the reverse transcriptase, and the env gene which codes for the envelope proteins. The transcription of retroviral message proceeds through a DNA intermediate, the provirus. The provirus represents a transcriptional unit that contains its own gene expression regulatory sequences within the long terminal repeats (26, 155).

Retroviruses are composed of about 60-70% protein, 30-40% lipid, 2-4% carbohydrate and 1% RNA (154). Purified EIAV contains structural polypeptides analogous to those of avian and murine type C-oncoviruses and include two envelope glycoproteins and four major internal non-glycosylated proteins (112, 125). The relative molecular weights of EIAV structural proteins in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) are 90,000 daltons and 45,000 daltons, respectively, for the two surface glycoproteins (gp), gp90 and gp45 and core proteins of 26,000, 15,000, 11,000 and 9,000 daltons, respectively (p26, p15, p11, p9) (8, 125). In addition to the six main structural proteins four minor polypeptides (p70, p61, p30 and p23) are revealed in SDS-PAGE (125). These minor polypeptides could be components of the virion reverse transcriptase or uncleaved precursors for the structural proteins.

The gag gene products are translated from a complete viral messenger RNA and processed from a polyprotein (NH2-p15-p26-p11-p9-COOH) to form the inner core of the virus (112, 125, 148). The core of the virus

encloses the ribonucleoprotein complex which is composed of viral RNA, the basic protein p11 and 6-10 copies of the reverse transcriptase. The most abundant structural protein is p26 which forms the core shell with the acidic protein p9. The fourth core protein is p15 which forms a continuous inner coat or mantle beneath the lipid bilayer (112, 125).

The virus glycoproteins are translated from a spliced messenger RNA that corresponds to the 3'-terminal end of the viral genome and are subsequently processed from a polyprotein precursor (42). The env gene, 2577 bases long, encodes the virion envelope glycoproteins in the order NH2-gp90-gp45-COOH (135). Partial digestion of purified virus with bromelain completely removed the two viral glycoproteins indicating their location on the surface of the virion (112). The major envelope glycoprotein (gp90) is heavily glycosylated (125, 135). In contrast, gp45 is a less glycosylated and highly hydrophobic glycoprotein that is associated with the lipid bilayer (125, 135). The spikes and knobs observed in mature EIA virions represent gp45 and gp90, respectively (112, 135, 158). Analogous to other enveloped virus systems, the major envelope glycoprotein of EIAV, gp90 probably mediates important functions in the virus replication cycle including host receptor recognition and may be the primary target for antibody-mediated virus neutralization (111, 112). Monoclonal antibodies specific for gp90 neutralize virus infectivity in cell culture (74). It is thus probable that antibodies to gp90 mediate virus neutralization in vivo.

Apart from the characteristic retroviral genes, gag, pol and env, the lentiviruses possess other open reading frames whose translation products may be related to their unique biology. The EIAV genome contains three

such open reading frames S1, S2 and S3 (135) that may have functions analogous to those documented for HIV (19).

Hybridization studies using cDNA (17, 133) or molecularly cloned EIAV probes (165) have clearly demonstrated that EIAV is an exogenous mammalian retrovirus. Moreover, genetic comparisons of EIAV with other lentiviruses have demonstrated that EIAV is more closely related to this subfamily of retroviruses than to the oncoviruses (20, 137, 165). Furthermore, group specific and interspecies determinants of EIAV cross react with human and ovine lentiviruses in immunological assays (109, 137, 165). Sera from horses infected with EIAV recognize the major core protein, p24 (109) and the major envelope glycoprotein, gp120 (137) of HIV-1. The genetic comparisons of EIAV with prototype lentiviruses have provided more definitive evidence for the assignment of EIAV as a lentivirus and confirm earlier assignments that were based on serological and morphological similarities (6, 15, 56, 57, 109).

INTERACTION OF LENTIVIRUSES WITH THEIR HOSTS.

The initial event in the life-cycle of viruses is the attachment to specific receptors on the cell surface (150). In many cases, this event is a major determinant of virus tropism in pathogenesis (141). Different viruses utilize different specialized cell structures as receptors (37): these viral receptors have important host functions (25) and in most cases internalize specific ligands by receptor-mediated endocytosis (54). Lentiviruses infect a variety of cells wherein they cause lytic or persistent infections depending on the virus and the cell type. The prototype lentivirus visna virus undergoes an acute lytic cycle in cultured sheep

choroid plexus (SCP) cells in which the yield of viral progeny is high. In the same cell type, CAEV causes a non-lytic infection in which the yield of progeny virus is 1000-fold lower than that of visna virus (116). The cell culture-adapted Wyoming (98) strain of EIAV (prototype EIAV) causes persistent infections in equine fibroblasts including those derived from primary fetal equine kidney cell cultures (16). The prototype EIAV has been observed to cause cytopathology in fetal donkey dermal cells (McManus, unpublished results).

In contrast to the animal lentiviruses, HIV does not infect cultured fibroblasts and epithelial cells (95). The restriction of HIV to infect these cells is predominantly due to the absence of surface receptors. Transfection of cultured fibroblasts with a molecular clone of HIV (ARV-2) showed that the virus can replicate in cells from several mammalian species; however, the virus replicated better in human than in mouse derived fibroblasts (96). Transfection of tumor cells with the isolated CD4 complementary DNA demonstrated that CD4 expression was the sole requirement for virus to infect target cells (105). Although the lentiviruses infect a variety of cultured fibroblasts, studies with the ruminant lentiviruses indicate that the target cells in the persistently infected animal are the monocyte-macrophages and not the fibroblasts (116, 117). However, fibroblasts and possibly other cell types, may be more involved in the pathogenesis of persistent lentiviral infections than previously thought. Indeed, the prototype EIAV can be reisolated in primary fetal equine kidney cell cultures even after multiple passages in Shetland ponies (122).

The mononuclear phagocytic system (monocytes and macrophages) is

considered to be one of the primary non-specific defense mechanisms against foreign agents including viruses. In addition, these cells are involved in the induction, regulation and amplification of the immune system, thus playing a central role in eliminating viruses from the infected host. The ability of viruses to infect these cells may be crucial to their persistence since latently infected cells can exist in the presence of neutralizing antibodies. Such latently infected cells may, in a manner analogous to the Trojan horse, serve as reservoir and/or vehicle for virus dissemination in an immunologically responsive host (128). Thus, viruses that are capable of invading these cells can very readily evade the immune system. Furthermore, these cells can take in viruses by specific (virus receptors) as well as by non-specific (Fc-receptors) mechanisms, thus making them a favorable target cell population for many viruses.

A number of viruses have a predilection for the mononuclear phagocytic system wherein virus replication, persistence, and spread in the host occurs as a result of the infection of these cells. The monocyte-macrophage series has been implicated in the pathogenesis of lentivirus infections because viral antigens, viral genomes and/or virus replication can be demonstrated in this cell population by a variety of techniques including immunofluorescence, immunocytochemistry, in situ hybridization, and electron microscopy (86, 106, 128). Moreover, infectious virus can be isolated from these cells by cocultivation with susceptible cell lines and macrophages readily support virus replication in vitro (5, 51, 95, 116)

The unique biology of lentiviruses can best be exemplified by HIV

which infects a number of cell types and causes an immunodeficiency in humans that becomes clinically apparent years after the initial infection. The virus infects a number of cell types that bear the CD4 molecule on the surface. HIV infects T4 lymphocytes where it causes lytic or persistent infections (10, 14, 38, 48, 72, 85, 105, 129). The virus also replicates in non-T lymphoid cell types including normal blood-derived monocytes, macrophages, EBV-transformed B-lymphocytes, malignant glioma cells and human monocytoid cell lines (7, 21, 93, 95, 110, 119). The virus has been isolated from human peripheral blood leukocytes (PBL) and alveolar macrophages of AIDS patients and causes a persistent infection in cultured PBLs (21, 49, 93, 95, 119). This non-cytopathic infection of monocytes and macrophages is probably due to a lower surface density of CD4 molecules: the CD4 glycoprotein appears to be an integral component in HIV-induced cell killing. However, as these cells harbor HIV proviral DNA they may serve as a reservoir for virus persistence in the host (58, 86, 93, 143). Isolates of HIV may differ in their ability to productively infect human cells (46). As in the animal lentiviruses, the factors involved in the restriction of virus replication in vivo are not known but may include cytokines e.g., interleukins, interferons and intercurrent pathogens e.g., EBV, cytomegalovirus and hepatitis B infections.

In contrast to the high yield of viral progeny in vitro, the replication of lentiviruses is severely restricted in vivo and with the exception of EIA where high levels of plasma viremia occur during clinical disease episodes, cell-free virus is not detectable in the other lentivirus infections. In EIA, macrophages are the only cells identified which harbor the virus and support replication of the wild-type virus (88, 106,

149). In some asymptomatic horses infectious virus cannot be demonstrated in the serum or whole blood but the infection can be transmitted with washed leukocytes (31). Thus, in a manner analogous to the other lentiviruses, EIAV probably infects many cell types including promyelocytes, monocytes and macrophages (51, 95, 116). Lentivirus replication may be intricately associated with the functional status of these cells. In visna, only a small proportion of monocytes is infected and virus replication is associated with the maturation of monocytes to macrophages (51, 52). Virus replication appears to be restricted at the transcription level (52, 128) apparently through a complex interaction involving infected monocyte/macrophages, T-lymphocytes and a lentivirus-induced interferon (118). In infected sheep, virus replication is repressed in many cell types including the monocyte/macrophages in tissue fluids with neutralizing antibody (128). In a similar manner, HIV genome expression appears to be repressed and high levels of genome expression are only observed after prolonged mitogenic stimulation of mononuclear cells from AIDS patients. The repressed state of lentivirus replication in vivo suggests that these viruses can persist in the face of a vigorous immune response as most infected cells are not producing viral antigens and escape detection by the immune surveillance mechanism.

As with other retroviruses, lentiviruses can persist in susceptible cells by integrating their DNA proviruses into host cell DNA (155). Once integrated, the provirus is replicated and duplicated equally to daughter cells along with the host DNA, thus ensuring survival of the viral genome. In lentivirus infections, both integrated and unintegrated proviruses have been demonstrated in cultured cells and in cells or tissues

from infected individuals (17, 65, 133, 142, 164). The replication cycle of visna virus in cultured cells occurs without provirus integration (65). The biological significance of the level of provirus integration observed in lentivirus infections is not known but may be related to the exogenous nature of this subfamily of retroviruses (62).

ANTIGENIC VARIATION

The survival of parasites in their target hosts is continuously challenged by the immunological mechanisms involved in host protection. Faced with these immunological barriers, parasites have improvised growth strategies whose sum enable them to persist in their hosts. The repertoire of these growth strategies includes: partial or complete genetic changes involving parasite surface structures; growth in cells of the immune system including lymphocytes, monocytes and macrophages; growth in parts of the body with less vigorous immunological surveillance (e.g. CNS); and an inability to express parasite antigens for long periods, i.e. latency. The games that parasites play are best exemplified by a consideration of the survival mechanisms displayed by the African trypanosomes and the influenza virus.

The antigenic properties of the African trypanosomes are due to a single glycoprotein, the variant surface glycoprotein (VSG). Each trypanosome contains over 100 VSG genes whose products are serologically and biochemically different (11). Antigenic variation, mediated by sequential expression (via DNA recombination) of the VSG genes, is the primary mechanism for the survival of trypanosome population in immunologically responding hosts.

Viruses with RNA genomes evolve by a variety of means. These include reassortment of gene segments, recombination between homologous nucleic acid molecules, point mutations, sequence repetition, deletion and inversion. Reassortment of virus genomes occurs with viruses that have segmented genomes regardless of their strandedness or complement of strand segments (124). If cells are infected with two related viruses, there can be an exchange of homologous gene segments with the production of different stable reassortants.

Two distinct kinds of antigenic variation have been demonstrated in influenza A viruses; antigenic shift and antigenic drift. Antigenic shift in type A influenza virus occurs infrequently when a "new" virus suddenly appears with hemagglutinin (HA) and/or neuraminidase (NA) molecules of a subtype different to those of the virus circulating before the new virus appeared. Antigenic shift is thought to occur when: 1) genetic reassortment occurs between human and animal or human viruses; and 2) animal or bird influenza A viruses undergo mutations which allow them to become infectious for humans (3).

At more frequent intervals, minor changes termed antigenic drift, take place in one or both of the surface glycoproteins (HA and NA). Antigenic drift in influenza A virus occurs by point mutations in the HA and NA genes that lead to a gradual accumulation of amino acid sequence changes that alter the epitopes in such a way that they no longer are recognized by existing specific immune effector mechanisms. Although each episode of drift may be minor, the effects are additive and over a period of several years result in a virus showing a considerable degree of antigenic difference from the original pandemic virus. The point

mutations are predominantly in the amino terminal end of the HA gene and cluster to four antigenic sites in the native HA polypeptide. Antigenic analysis of HA with monoclonal antibodies (MAbs) and polyspecific antisera revealed that epidemiologically significant strains sustain point mutations at two or more antigenic sites; a large number of mutations are required for complete resistance to polyspecific antisera (3, 12, 146, 157, 167).

In visna, new virus strains do not successively replace parental virus; both the inoculum strain and the variant strains can be isolated from peripheral blood leukocytes simultaneously. The new strains of visna virus have been shown to arise by point mutations in the env gene which result in structural alterations in the major envelope glycoprotein, gp135 (24, 138). In most cases, visna virus persists and spreads without the emergence of new virus variants (97, 153). Besides the repressed state of visna virus genome expression in monocyte/macrophages, visna virus can infect these cells in the presence of "neutralizing" antibodies indicating that antigenic variation is not critical for the survival of this virus in immunocompetent hosts (84).

In chronic EIA there are cycles of virus replication in which virus can be isolated from plasma. Neutralization studies using polyclonal sera obtained from rabbits immunized with purified virus demonstrated the existence of antigenic drift in EIAV (91). Each new virus isolate from a cycle is refractory to neutralization by antibody that neutralized previous EIAV isolates (91, 123, 136). Recent studies have further documented that viruses recovered from sequential febrile episodes are structurally and genetically unique (127, 136). Western blot analyses of EIAV isolates

revealed that antigenic alterations occurred predominantly in the major envelope glycoprotein, gp90 (74). The survival of EIAV in immunologically responsive hosts appears to be mediated by antigenic variation. From this standpoint, it is imperative to determine the extent to which this virus varies and exists in susceptible animal populations before any immunoprophylactic or chemoprophylactic intervention can be contemplated.

The contention that lentiviruses demonstrate rapid genomic changes has recently been extended by results from the analysis of HIV isolates obtained from the same or different individuals (64, 163). Analysis of HIV-1 isolates from different geographical regions revealed extensive genomic variation particularly within the region encoding the viral envelope glycoprotein (4, 63, 162, 163). A similar variability has been observed in viruses sequentially isolated from persistently infected individuals (64). Viruses isolated from one individual are more genetically related to each other than to viruses isolated from other individuals and may therefore have evolved in parallel from a common progenitor (64, 163); virus interference may exclude superinfection with many strains of HIV. The genetic variability of HIV results largely from small duplications, insertions or deletions as well as from numerous point mutations in the envelope gene (63, 63, 162). Within the env gene, hypervariable regions are interspersed with regions of strong conservation (131, 147, 162). Complementing the sequence data, serological studies have shown that HIV-1 isolates are antigenically heterogeneous (22, 103, 159). HIV-2 is genetically and antigenically more closely related to the simian immunodeficiency virus (SIV) than to HIV-1 (23, 60). Molecular and

serological data indicate that HIV is as highly mutable as the animal lentiviruses (24, 127).

Like RNA viruses in general, lentiviruses have high mutation frequencies due to the lack of error correcting mechanisms in genome replication (132). The estimated error rate for RNA replicases lies between 10^{-3} and 10^{-4} (71, 132) and is therefore of the order of 10^{4}- to 10^{6}-fold greater than the mutation rates of stable DNA genomes (69, 71). Replication of RNA is therefore an intrinsically "noisy" process (132). The noisiness of RNA replicases means that the genomes of RNA viruses are not monolithic, but are constantly evolving irrespective of the attested homogeneity of any starting virus population (69). Analyses of lentivirus genomes by T1-oligonucleotide mapping, Southern blot, restriction enzymes and nucleotide sequencing reveal that genomic variations range from slight to rather extensive changes; such changes are observed with highest frequency in the env gene and are compatible with the expected mutational rates of RNA viruses (64, 113, 136, 163). These genomic changes may result from errors associated with reverse transcription and from genetic recombination during mixed infections with related viruses.

The viral glycoproteins are involved in host-cell recognition and may be the primary targets for antibody mediated neutralization. The role of envelope glycoproteins in eliciting neutralizing antibodies has been established for a number of viruses including feline leukemia virus gp-70 (121), influenza virus hemagglutinin (156), herpes simplex virus glycoprotein-D (33), rabies virus glycoprotein-G (161), human immunodeficiency virus gp120 (160) and EIAV gp90 (74). Thus from an immunoprophylactic point of view, knowledge of the structure, immunogenicity, antigenicity

and variability of the surface glycoproteins of lentiviruses is imperative.

Specific antibodies exert antiviral effects via several distinct mechanisms. First, intrinsic neutralization may occur. This mechanism involves loss of virus infectivity mediated by the direct binding of antibody to neutralization antigenic sites. In some cases, bound antibody may mediate neutralization only in the presence of complement or anti-globulin. Second, extrinsic neutralization may result in the loss of virus infectivity due to an inhibition of virus adsorption which results from antibody binding at or nearby the viral attachment proteins. In enveloped viruses, antibodies attached to the envelope glycoproteins can also mediate neutralization by complement-mediated lysis of the virus membrane. A third mechanism, pseudoneutralization, which may function under certain conditions, e.g., at equivalent concentrations of virus and antibody, results in loss of virus infectivity by promoting formation of lattice like aggregates of infectious virions. Low affinity antibodies e.g., IgM and early IgG tend to have higher levels of "sensitized" virus. Moreover, the cell type and the environmental conditions may also contribute to this phenomenon (43, 104, 111, 120).

Antibodies can neutralize poliovirus by inducing conformational changes in the capsid (44, 45, 99). These changes may result in an alteration of molecular functions including loss of efficient virus adsorption to host cells. Cross-linkage of pentameric subunits of the virus capsid (76) or aggregation of virus particles (2) result in poliovirus neutralization. Conformational alterations in the viral capsid may result in the physical loss of viral genome causing irreversible loss of infectivity as exemplified by foot-and-mouth disease virus (104).

Although the attachment of virus to host cells can be prevented by neutralizing antibody, e.g. reovirus infection of L-cells (94), in most cases, the binding of neutralizing antibodies to virions does not inhibit virus attachment to host cells. This may be because the cellular binding site is distinct from the epitopes involved in neutralization, e.g. with influenza virus (134). Antibodies that neutralize influenza virus do not inhibit attachment, penetration, uncoating of virus or transport of the uncoated RNA into the nucleus. Moreover, the mechanism of virus neutralization may depend on the antibody isotype. Thus, monomeric IgG probably neutralizes influenza virus by inhibiting the function of the viral transcriptase (130), while polymeric IgA and IgM may neutralize the virus by steric hinderance of the viral receptor "pocket" in the HA (151, 152). Antibody induced conformational changes may result in the neutralization of enveloped viruses by inhibition of a low-pH mediated fusion reaction between the membrane of the virus particle and that of a prelysosomal endosome, thus impairing the entry of viral cores into the cytosol as has been shown with the West Nile virus (55). The different modes of action of the various immunoglobulin classes, the different viruses and the different cell systems used by many investigators may help to explain why virus neutralization is still a controversial subject.

It is generally accepted that immune selection pressure operates in the selection of virus mutants during antigenic drift. Antigenic drift has been mimicked in the laboratory by the propagation of viruses in the presence of low levels of neutralizing polyclonal antisera or monoclonal antibodies. Such studies have been conducted for a number of human and animal viruses including the human influenza A viruses (53), poliovirus

type 1 (41), herpes simplex virus (70), foot-and-mouth disease virus (75), and visna virus (115). A general outcome of propagating these viruses in the presence of neutralizing antibodies has been the emergence of virus variants that resisted neutralization by the selecting antibody. Chronic EIA, characterized by recurrent febrile episodes, is thought to be induced by novel virus variants (90) that probably arise through random virus genome mutations and antibody selection pressure. Thus, generation of neutralization escape mutants of EIAV in cell culture would aid in substantiating the role of antibody in antigenic drift. Antigenic and genetic analysis of neutralization escape mutants may identify the changes associated with the ability of virus variants to elude host immunological controls.

In infections of horses with EIAV, serum neutralizing antibodies have not been detected earlier than 45 days after infection and peak titers occurred later in the course of the disease (89). This raises the question of the nature of both specific and non-specific immunological controls operative in the recovery from acute EIA in the absence of demonstrable neutralizing antibody. Furthermore, the effectiveness of neutralizing antibodies is periodically circumvented by the ability of the virus to change antigenically (90) thus allowing virus mutants to replicate until immunological controls are reestablished. Eventually, horses with chronic EIA cease to have recurrent febrile episodes but remain inapparently infected for life. It is not clear how much antigenic relatedness exists between viral isolates. If present, it is apparently minor and inadequate to stimulate cross-protection by antibody (90, 91).

Most horses mount a vigorous humoral and cell-mediated immune

(CMI) response to most if not all EIA antigens and CMI may be the principal mechanism involved in long term host protection against recurrent EIA (108). Indeed, when asymptomatic horses were treated with dexamethasone or cyclophosphamide, viremia and clinical disease occurred (92). The time interval between treatment and disease expression was too short to be explained by an effect on antibody levels. Furthermore, horses with chronic EIA resisted infection by serotypes to which they had no demonstrable neutralizing antibody, which suggests that immunological factors other than antibody participate in protecting horses from recurrent febrile episodes (90). In viral infections, a T-cell mediated immune response may be the most important regulator of the host to infection. Thus, the induction of a CMI response may be an important component of the overall immunity directed towards viruses. Although definitive mechanisms of CMI responses have not been obtained in EIA, evidence from influenza virus indicates that viral envelope glycoproteins, as well as internal viral proteins, are involved in the generation of serotype-specific and cross-reactive cytotoxic T lymphocytes (CTLs), respectively (1). Although the internal viral proteins are involved in the generation of cross-reactive CTLs, vaccination of mice with recombinant vaccinia virus containing the nucleoprotein (NP) of influenza virus did not induce complete protection (167) indicating that HA is required for complete protection to occur; this implies that strain specific neutralizing antibodies and CTLs are important in the primary defense against influenza virus infection.

A major challenge in the control of viruses that evolve as rapidly as EIAV is development of effective vaccination protocols. A combination of

molecular and genetic studies of the lentiviruses has shed light on some unique properties of these viruses, especially their ability to evolve rapidly. However, fundamental aspects of the mechanisms by which these viruses are transmitted from host to host and how they persist within immunoresponsive hosts need to be identified. A better understanding of the pathogenetic mechanisms of EIAV and the immune responses important in host protection is desirable for a rational assessment of strategies for vaccine development.

RESEARCH OBJECTIVES.

Equine infectious anemia is a disease of horses that occurs throughout the world. The disease tends to have a recurrent course and infected horses carry the virus for life. Recurrent episodes of EIA are thought to be due to the emergence of novel variants of EIAV that escape immunological controls existent at the time of their emergence. Recent studies have shown that EIAV mutants readily evolve in infected animals. To date, no form of chemotherapeutic or immunological intervention in EIA is in use. For any of these measures to be anticipated or even attempted, a detailed analysis of the mechanism(s) involved in the persistence of EIAV needs to be achieved. Thus, an assessment of the repertoire of serotypes of EIAV and their relatedness to each other is central to any attempt to control this important viral disease of horses by vaccination. To address this issue, virus variants were generated in Shetland ponies. For this study, the cell culture adapted Wyoming strain of EIAV (98) was used at its second passage in Shetland ponies to infect a series of third passage recipients. EIAV isolates obtained from these ponies during febrile episodes were characterized by serological and biochemical methods.

The following is an outline of the research objectives:

1. Generate a panel of EIAV variants in Shetland ponies.
    a. Isolate EIAV from acute-phase plasma by end-point dilution in fetal equine kidney cell cultures.
    b. Characterize the virus isolates in cross-neutralization assays using a panel of EIA convalescent sera and rabbit hyperimmune sera

against lentil-lectin affinity purified EIAV glycoproteins.

c. Analyze these viral isolates in Western immunoblots and using a panel of MAbs and convalescent antisera.

d. Analyze viral glycoproteins by two-dimensional tryptic-peptide mapping.

2. Isolate neutralization escape mutants using neutralizing convalescent sera. Analyze these mutants in:

   a. Western blot using convalescent sera and MAbs.

   b. Neutralization by heterologous convalescent sera and the glycoprotein specific antisera.

3. Document the kinetics of anti-EIAV humoral responses during persistent infection.

4. Evaluate a single-step immunoblot assay for EIAV antibody and antigen detection, respectively.

REFERENCES CITED

1. ADA, G. C., JONES, P. D.: The immune response to influenza infection. Curr. Top. Microbiol. Immunol. **128**, 1-54 (1986).
2. ADRI, A. M., THORMAS, RAF-VRIJSEN, ALBERT, B.: Relationship between poliovirus neutralization and aggregation. J. Virol **59**, 479-485 (1986).
3. AIR, G. M., LAVER, W. G.: The molecular basis of antigenic variation in influenza virus. Adv. Virus Res. **31**, 53-103 (1986).
4. ALIZON, M., WAIN-HOBSON, L., MONTAGNIER, L., SONIGO, P.: Genetic variability of the AIDS virus: nucleotide sequence analysis of two isolates from African patients. Cell **46**, 63-74 (1986).
5. ANDERSON L. W., KLEVJER-ANDERSON, P., LIGGITT, H. P.: Susceptibility of blood-derived monocytes and macrophages to caprine arthritis-encephalitis virus. Infect. Immun. **41**, 437-840 (1983).
6. ARCHER, B. G., CRAWFORD, T. B., MCGUIRE, T. C., FRAZIER, M. E.: RNA-dependent DNA polymerase associated with equine infectious anemia virus. J. Virol. **22**, 16-22 (1977).
7. ASJO, B., IVHED, I., GIDLUND, M., FUERSTENBERG, S., FENYO, E. M., NILSSON, K., WIGZELL, H.: Susceptibility to infection by the AIDS virus correlates with T4 expression in monocytoid cell lines. Virology **157**, 359-365 (1987).
8. AUGUST, J. T., BOLOGNESI, D. P., FLEISSNER, E., GILDEN, R. V., NOWINSKI, R. C.: A proposed nomenclature for the virion proteins of oncogenic RNA viruses. Virology **60**, 595-601 (1974).

9. BARNETT, D.: Heat inactivation of equine infectious anemia virus in equine serum. Proc. 76th U.S. Anim. Hlth Assoc. Mtg pp. 231-236 (1972).

10. BARRE-SINOUSSI, F., CHERMAN, J. C., REY, F., NUGEYRE, M. T., CHAMARET, S., GRUEST, J., DAUGUET, C. AXLERBLIN, C., VEZINET-BRUN, F., ROUZIOUX, C., ROZENBAUM, W., MONTAGNIER, L.: Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science **220**, 868-871 (1983).

11. BOUILLANT, A. M. P., BECKER, S. A. W. E.: Ultrastructural comparison of oncovirinae (Type C), spumavirinae and lentivirinae: Three subfamilies of retroviridae found in farm animals. J. Nat. Cancer Inst. **72**, 1075-1084 (1984).

12. BORST, P. CROSS, G. A. M.: Molecular basis of trypanosome antigenic variation. Cell, **29**, 291-303 (1982).

13. BOTH, G. W., SLEIGH, M. J., COX, N. J. KENDAL, A. P. Antigenic drift in influenza virus H3 hemagglutinin from 1968 to 1980: Multiple evolutionary pathways and sequential amino acid changes at key antigenic sites. J. Virol. **48**, 52-60 (1983).

14. CASAREALE, D. C., STEVENSON, M., SAKAI, K., VOLSKY, D. J.: A human T-cell line resistant to cytopathic effects of the Human immunodeficiency virus (HIV). Virology **156**, 40-49 (1987).

15. CHARMAN, H. P., BLADEN, S., GILDEN, R. V., COGGINS, L.: Equine Infectious Anemia Virus: Evidence favoring classification as a retrovirus. J. Virol. **19**, 1073-1079 (1976).

16. CHEEVERS, W. P., ARCHER, B. G., CRAWFORD, T. B.: Charact-

erization of RNA from equine infectious anemia virus. J. Virol. **24**, 489-497 (1977).

17. CHEEVERS, W. P., WATSON, S. G., KLEVJER-ANDERSON, P.: Persistent infection by Equine infectious anemina virus: Asymmetry of nucleotide sequence reiteration in the integrated provirus of persistently infected cells. Virology **118**, 246-253 (1982).
18. CHEEVERS, W. P. McGUIRE, T. C.: Equine Infectious Anemia Virus: Immunopathogenesis and persistence. Rev. Infect. Dis. **7(1)**, 83-88 (1985).
19. CHEN, I. S. Y.: Regulation of AIDS virus expression. Cell **47**, 1-2 (1986).
20. CHIU, I. M., YANIV, A., DAHLBERG, J. E., GAZIT, A., SKUNTZ, S. F., TRONICK, S. R., AARONSON, A.: Nucleotide sequence evidence for relationship of AIDS retrovirus to Lentiviruses. Nature **317**, 366-368 (1985).
21. CLAPHAM, P. R., WEISS, R. A., DALGLEISH, A. G., EXLEY, M., WHITBY, D. HOGG, N.: Human immunodeficiency virus infection of monocytoid and T-lymphocytic cells: Receptor modulation and differentiation induced by phorbol ester. Virology **158**, 44-51 (1987)
22. CLAVEL, F., KLATZMANN, D. MONTAGNIER, L.: Deficient LAV-1 neutralizing capacity of sera from patients with AIDS or related syndromes. Lancet **1**, 879-880 (1985).
23. CLAVEL, F., GUETARD, D., BRUN-VEZINET, F., CHAMARET, S., REY, M. A., SANTOS-FERREIRA, M. O., LAURENT, A. G., DAN-GUET, C., KATLAMA, C., ROUZIOUX, C., KLATZMANN, D., CHAMPALIMUD, J. L., MONTAGNIER, L.: Isolation of a new human

retrovirus from West Africa patients with AIDS. Science **233**, 343-346 (1985).

24. CLEMENTS, J. E., PEDERSEN, F. S., NARAYAN, O., HASELTINE, W. A.: Genomic changes associated with antigenic variation of visna virus during persistent infection. Proc. Natl. Acad. Sci. U.S.A **77**, 4454-4458 (1980).

25. CO, M. S., FIELDS, B. N., GREENE, M. I.: Viral receptors serving host functions. pp.126-131. In Concepts in Viral Pathogenesis, Vol 2. Springer-Verlag, NY (1986).

26. COFFINS, J., HAASE, J. A., LEVY, A., MONTAGNIER, L., OROSZLAM, S., TEICH, N., TEMIN, H., TOYOSHINIA, K., VARMUS, H. VOGT, P., WEISS, R.A.: What to call the AIDS virus? Nature **321**, 10 (1986).

27. COFFIN, J.: Structure of the retroviral genome. pg 261-368. In Molecular Biology of Tumor Viruses: RNA Tumor viruses. 2nd Edition, Weiss, R., Teich, N., Varmus, H., and Coffin, J. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984).

28. COGGINS, L. NORCROSS, N. L.: Immunodiffusion reaction in equine infectious anemia. Cornell Vet. **60**, 330-335 (1970).

29. COGGINS, L., NORCROSS, N. L. NAUSTRAUM, S. R.: Diagnosis of equine infectious anemia by immunodiffusion test. Am. J. Vet. Res. **33**, 11-18 (1972).

30. COGGINS, L.: Mechanism of viral persistence in equine infectious anemia. Cornell Vet. **65**, 143-151 (1972).

31. COGGINS, L., KEMEN, M. J.: Inapparent carriers of equine infectious anemia (EIA) virus. Equine infectious anemia workshop

proceedings, 14-22. 4th Int. Conf. on Equine Infectious Diseases, Ky, Grayson Foundation, 1976.

32. COGGINS, L. MATHEKA, H. P., CHARMAN, H. P.: Radioimmunoassay for equine infectious anemia virus antigen and antibody. J. Eq. Med. Surg. **1**, 351-358 (1978).

33. COHEN, G. H., DIETZSCHOLD, B., PONCE DE LEON, M., LONG, D., GOLUB, E., VARRICHIO, A., PEREIRA, L., EISENBERG, R.: Localization and synthesis of an antigenic determinant of herpes simplex virus glycoprotein D that stimulates production of neutralizing antibody. J. Virol. **49**, 102 (1984).

34. CORK, L. C., HADLOW, W. J., CRAWFORD, T. B., GORHAM, J. R. PIPER, R.C.: Infectious leukoencephalomyletis of young goats. J Infect. Dis. **129**, 134-141 (1974).

35. CRAWFORD, T. B., MCGUIRE, T. C., HENSON, J. B.: Detection of equine infectious anemia in vitro by immunofluorescence. Arch. Virol. **34**, 332-339 (1971).

36. CRAWFORD, T. B., CHEEVERS, W. P., KLEVJER-ANDERSON, P., MCGUIRE, T.C.: Equine Infectious Anemia: Virion characteristics, virus-cell interaction and host responses. In Persistent Viruses. pp. 727-749. ICN-UCLA Symposium on Molecular and Cellular Biology, vol, 2. Stevens, J., Todaro, G. and Fox, C.F. (eds), Academic Press, NY (1978).

37. CROWELL, R. L., HSU, K-H, L.: Isolation of cellular receptors. pp. 117-125. In Concepts in Viral Pathogenesis vol 2. Springer-Verlag, NY (1986).

38. DALGLEISH, A. G., BEVERLERY, P. L. C., CLAPHAM, P. R.,

CRAWFORD, D. H., GREAVES, M. F., WEISS, R. A.: The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature **312**, 763-767 (1984).

39. DANIEL, M. D., KING, N. W., LEVTIN, N. L., HUNT, R. D., SEGHAL, P. K., DESROSIERS, R. C.: A new type D retrovirus isolated from macaques with immune-deficiency syndrome. Science 227, 177-181 (1984).

40. De BOER, G. F.,: Zwoegerziekte virus, the causative agent for progressive interstitial pneumonia (maedi) and meningo-leukoencephalitis (visna) in sheep. Vet. Res. **18**, 15-25 (1975).

41. DIAMOND, C. D., JAMESON, B. A., BONIN, J., KOHARA, M., ABE, S., ITOH, H., KOMATSU, T., ARITA, M., KUGE, S., NOMOTO, A., OSTERHAUS, A. D. M. E., CRAINIC, R., WIMMER, E.: Antigenic variation and resistance to neutralization in poliovirus type 1. Science **229**, 1090-1093 (1985).

42. DICKSON, C., EISENMAN, R., FAN, H., HUNTER, E., TEICH, N.: pg 513-648. In Molecular Biology of Tumor Viruses: RNA Tumor viruses. 2nd Edition, Weiss, R., Teich, N., Varmus, H., and Coffin, J. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984)

43. DIMMOCK, N. J.: Mechanisms of neutralization of animal viruses. J. Gen. Virol. **65**, 1015-1022 (1984).

44. EMINI, E. A., KAO, S. Y., LEWIS, A. J., CRAINIC, R., WIMMER, E.: Functional basis of poliovirus neutralization determined with monospecific neutralizing antibodies. J. Virol. **46**, 466-474 (1983a).

45. EMINI, E. A., OSTAPCHUK, P., WIMMER, E.: Bivalent attachment of antibody onto poliovirus leads to conformational alteration and

neutralization. J. Virol. **48**, 157 (1983b).

46. EVANS, L. A., MCHUGH, T. M., STITES, D. P., LEVY, J. A.: Differential ability of human immunodeficiency virus isolates to productively infect human cells. J. Immunol. **138**, 3415-3418 (1987).

47. FENNER, F.: The classification and nomenclature of viruses. Intervirology **6**, 1-12 (1975).

48. FOLKS, T., POWELL, D., LIGHTFOOTE, M. M., BENN, S., MARTIN, M. A., FAUCI, A. S.: Induction of HTLV-3/LAV from a nonvirus producing T-cell line: Implications for latency. Science **231**, 600-602 (1986).

49. GARTNER, S., MARKOVITS, P., MARKOVITS, D. M., KAPLAN, M. H., GALLO, R. C., POPOVIC, M.: The role of mononuclear phagocytes in HTLV-3/LAV infection. Science **233**, 215-219(1986).

50. GELDERBLOM, H. R., HAUSMANN, E. H. S., OZEL, M., PAULI, G., KOCH, M. A.: Fine structure of Human immunodeficiency virus (HIV) and immunolocalization of structural proteins. Virology **156**, 171-176 (1987).

51. GENDELMAN, H. E., NARAYAN, O., MOLINEAUX, S., CLEMENTS, J. E. GHOTIB, Z.: Slow, persistent replication of lentiviruses: Role of tissue macrophages and macrophage precursors in bone marrow. Proc. Natl. Acad. Sci. U.S.A **82**, 7086-7090 (1985).

52. GENDELMAN, H. E., NARAYAN, O., KENNEDY-STOSKOPF, S., KENNEDY, P. G. E., GHOTIB, Z., CLEMENTS, J. E., STANELY, J., PEZESHKAPOUR, G.: Tropism of sheep lentiviruses for monocytes: Susceptibility to infection and virus gene expression increase during maturation of monocytes to macrophages. J. Virol. **58**, 67-74 (1986).

53. GERHERD, W., WEBSTER, R. G.: Antigenic drift in influenza A viruses. 1. Selection and characterization of antigenic variants of A/PR/8/34 (H0N1) influenza virus with monoclonal antibodies. J. Exp. Med. **148**, 383-392 (1978).

54. GOLDSTEIN, J. L., BROWN, M. S., ANDERSON, R. G. W., RUSSELL, D. W., SCHNEIDER, W. J.: Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. Ann. Rev. Cell Biol. **1**, 1-39 (1985).

55. GOLLINS, S. W., PORTERFIELD, J. S.: A new mechanism for the neutralization of enveloped viruses by antiviral antibody. Nature **321**, 244-246 (1986).

56. GONDA, M. A., CHARMAN, H. P., WALKER, J. L., COGGINS, L.: Scanning electron microscopy study of EIAV. Am. J. Vet. Res. **39**, 431-440 (1978).

57. GONDA, M. A., WONG-STAAL, F., GALLO, R. C., CLEMENTS, J. E., NARAYAN, O., GELDEN, R. V.: Sequence homology and morphologic similarity of HTLV-3 and visna virus, a pathogenic lentivirus. Science **227**, 173-177 (1985).

58. GYORKEY, F., MELNICK, J., SINKOVICS, J., GYORKEY, P.: Retrovirus resembling HTLV-3 in macrophages of patients with AIDS. Lancet **1**, 106 (1985).

59. GUDNADOTTIR, M. PALSSON, P.: Transmission of maedi by inoculation of a virus grown in tissue culture from maedi-affected lungs. J. Inf. Dis. **117**, 1-6 (1967).

60. GUYADER, M. EMERMAN, M., SONIGO, P., CLAVEL, F., MONTAGNIER, L., ALIZON, M.: Genome organization and

transactivation of the human immunodeficiency virus type 2. Nature **326**, 662-669 (1987)

61. HAASE, A. T.: The slow infection caused by visna virus. Curr. Top. Microbiol. Immunol. **72**, 101-156 (1975).

62. HAASE, A. T.: Pathogenesis of lentivirus infections. Nature **322**, 130-136 (1986).

63. HAHN, B. H., GONDA. M., SHAW, G. M., POPOVIC, M., HOXIE, J., GALLO, R. C. WONG-STAAL, F.: Genomic diversity of the AIDS virus HTLV-3: different viruses exhibit greatest diversity in their envelope genes. Proc. Natl. Acad. Sci. U.S.A **82**, 4813-4817 (1985).

64. HAHN, B. H., SHAW, G. M., TAYLOR, M. E., REDFIELD, R. R., MARKHAM, P. D., SALAHUDDIN, S. Z., WONG-STAAL, F., GALLO, R. C., PARKS, E. S., PARKS, W. P.: Genetic variation in HTLV-3/LAV over time in patients with AIDS or at risk for AIDS. Science **232**, 1548-1554 (1986).

65. HARRIS, J. D., BLUM, H. B., SCOTT, J., TRAYNOR, B., VENTURA, P., HAASE, A.: Slow virus visna: Reproduction in vitro of virus from extrachromosomal DNA. Proc. Natl. Acad. Sci. U. S. A. **81**, 7212-7215 (1984).

66. HAWKINS, J. A., ADAMS, W. V. Jr., WILSON, B. H., ISSEL, C. J., ROTH, E. E.: Transmission of equine infectious anemia virus by Tabanus fuscicostatus. Am. J. Vet. Med. Ass. **168**, 63-64.

67. HENSON, J. B., McGUIRE, T. C.: Immunopathology of equine infectious anemia. Am. J. Clin. Path. **56**, 306-314 (1971).

68. HENSON, J. B., McGUIRE, T. C.: Equine infectious anemia. Prog. Med. Virol. **18**, 143-159 (1974).

69. HOLLAND, J., SPINDLER, K., HORODYSKI, F., GRABAU, E., NICHOL, S., VANDEPOL, S.: Rapid evolution of RNA genomes. Science **215**, 1577-1585 (1982).

70. HOLLAND, T. C., MARLIN, S. D., LEVINE, M., GLORIOSO, J.: Antigenic variants of herpes simplex virus selected with glycoprotein-specific monoclonal antibodies. J. Virol. **45**, 672-682 (1983).

71. HOLLAND, J. J.: Continuum of change in RNA virus genomes. In Concepts in viral pathogenesis. Edited by Abner Louis Notkins and Michael B.A. Oldstone. Springer-Verlag New York Inc. pp. 137-143 (1984).

72. HOXIE, J. A., HAGGARTY, B. S., RACKOWSKI, J. L., PILLSBURY, N., LEVY, J. A.: Persistent noncytopathic infection of normal human T lymphocytes with AIDS-Associated Retrovirus. Science **229**, 1400-1402 (1985).

73. HULL, R., COVEY, S. N.: Genome organization and expression of reverse transcribing elements: Variation and a Theme. J. Gen. Virol. **67**, 1751-1758 (1986).

74. HUSSAIN, K. A., ISSEL, C. J., SCHNORR, K. L., RWAMBO, P. M., MONTELARO, R. C.: Antigenic analysis of equine infectious anemia virus (EIAV) variants using monoclonal antibodies: epitopes of glycoprotein 90 (gp90) of EIAV stimulate neutralizing antibodies. J. Virol. **61**, 2956-2961 (1987).

75. HYSLOP, N. S. G.: Isolation of variant strains of foot-and-mouth disease virus propagated in cell cultures containing antiviral sera. J. Gen. Microbiol. **4**, 135 (1965).

76. ICENOGLE, J., SHIWEN, H., DUKE, G., GILBERT, S., RUECKERT, R., ANDEREGG, J.: Neuralization of poliovirus by monoclonal antibody: kinetics and stoichiometry. Virology **127**, 412-425 (1983).

77. ISSEL, C. J., COGGINS, L.: Equine infectious anemia: Current knowledge. Am. J. Vet. Med. Ass. **174**, 727-733 (1979).

78. ISSEL, C. J., ADAMS, W. V. Jr.: Serologic survey for equine infectious anemia virus in Louisiana. Ame. J. Vet. Med. Ass. **174**, 286-288 (1979).

79. ISSEL, C. J., FOIL, L. D.: Studies on equine infectious anemia virus transmission by insects. Ame. Vet. Med. Ass. **184** (3), 293-297 (1984).

80. KANKI, P. J., ALROY, J., ESSEX, M.: Isolation of T-lymphotropic retrovirus related to HTLV-3/LAV from wild-caught African green monkeys. Science **230**, 951-954 (1985).

81. KEMEN, M. J. Jr., COGGINS, L.: Equine infectious anemia: Transmission from infected mares to foals. Ame. J. Vet. Med. Ass. 161, 496-499 (1972).

82. KEMENY, L., MOTT, L., PEARSON, J.: Titration of equine infectious anemia virus. Effect of dosage on incubation time and clinical signs. Cornell Vet. **61**, 688-695 (1971).

83. KENNEDY, R. C., EKLUND, C. M., LOPEZ, C., HADLOW, W. J.: Isolation of virus from lungs of Montana sheep affected with progressive pnuemonia. Virology, **35**, 483-484 (1968).

84. KENNEDY-STOSKOPF, S., NARAYAN, O.: Neutralizing antibody to visna virus: mechanism of action and possible role in virus persistence. J. Virol. **59**, 37-44 (1986).

85. KLATZMANN, D., BARRE-SINOUSSI, F., NUGEYRE, M. T., DAUGUET, C., VILMER, E., GRISCELLI, C., ROUZIOUX, C., GLUCKMAN, J. C., MONTAGNIER, L.: Selective tropism of lymhadenopathy associated virus (LAV) for helper-inducer T lymphocytes. Science **225**, 59-63 (1984).

86. KOENIG, S., GENDELMAN, H. E., ORENSTEIN, J. M. DAL CANTO, M. C., PEZESHKPOUR, G. H., YUNGBLUTH, M., JANOTTA, F., ASKSAMIT A., MARTIN, M. A., FAUCI, A. S.: Detection of AIDS virus in macrophages in brain tissue from patients with encephalopathy. Science **233**, 1089-1093 (1986)

87. KONO, Y.: Viremia and immunological responses in horses infected with equine infectious anemia virus. Natl. Inst. Anim. Hlth. Quart. (Tokyo) **8**, 1-9 (1969).

88. KONO, Y., YOSHINO, T., FUKUNAGA, Y.: Growth characteristics of equine infectious anemia virus in horse leukocyte cultures. Arch. Virol. **30**, 252-256 (1970).

89. KONO, Y., KOBAYASHI, K., FUKUNAGA, Y.: Serological comparison among various strains of equine infectious anemia virus. Arch. Virol. **34**, 202-208 (1971).

90. KONO, Y., KOBAYASHI, K., FUKUNAGA, Y.: Antigenic drift of equine infectious anemia virus in chronically infected horses. Arch. Virol. **41**, 1-10 (1973).

91. KONO, Y.: Reccurences of equine infectious anemia. Approaches to understanding of the mechanisms. In Proc. 3rd Int. Conf. Equine Infectious Diseases, pp. 175-186. Basel: S. Karger (1973).

92. KONO, J., HIRASAWA, K., FUKUNAGA, Y., TANIGUCHI, T.

Recrudescence of equine infectious anemia by treatment with immunosuppressive drugs. Natl. Inst. Anim. Hlth. Quart. **16**, 8-15.

93. KOYANAGI, Y., MILES, S., MITSUYASU, R. T., MERRILL, J. E., VINTERS, H. V., CHEN, I. S. Y.: Dual infection of the central nervous system by AIDS viruses with distinct cellular tropisms. Science **236**, 819-822 (1987).

94. LEE, P. W. K., HAYES, E. C., JOKLIK, W. C.: Protein s1 is the reovirus attachment protein. Virology **108**, 156-163 (1981).

95. LEVY, J. A., SHIMABUKURO, J., MCHUGH, T., CASAVANT, C., STITES, D., OSHIRO, L.: AIDS-associated retrovirus (ARV) can productively infect other cells besides human T helper cells. Virology **147**, 441-448 (1985).

96. LEVY, A. L., CHENG-MAYER, C., DINA, D., LUCIW, P. A. AIDS retrovirus (ARV-2) clone replicates in transfected human and animal fibroblasts. Science **232**, 998-1001 (1986).

97. LUTLEY, R., PETURSSON, G., PALSSON, P. A., GEORGSSON, G., KLEIN, J., NATHANSON, N.: Antigenic drift in visna virus: virus variation during long term infection of Icelandic sheep. J. Gen. Virol. **64**, 1433-1440 (1983).

98. MALMQUIST, W. A., BARNETTE, D., BECVAR, C. S.: Production of equine infectious anemia virus antigen in a persistently infected cell line. Arch. Virol. **42**, 361-370 (1973).

99. MANDEL, B.: Neuralization of poliovirus: a hypothesis to explain the mechanism and one-hit character of the neutralization reaction. Virology **69**, 500-510 (1976).

100. MANDEL, B.: Interaction of viruses with neutralizing antibodies. In

Comprehensive Virology, vol. 15, pp. 37-121. Edited by H. Fraenkel-Conrat & R. R. Wagner. New York: Plenium Press (1979).

101. MANDEL, B.: Mechanism of virus neutralization. In Concepts in viral pathogenesis. Edited by Abner Louis Notkins and Michael B.A. Oldstone. Springer-Verlag New York Inc. pp. 32-38 (1984).

102. MATHEKA, H. D., COGGINS, L., SHIVELY, J. N., NORCROSS, N. L.: Purification and characterization of equine infectious anemia virus. Arch. Virol. **51**, 107-114 (1976).

103. MATHEWS, T. J., LANGOS, A. J., ROBEY, W. G., CHANG, N. T., GALLO, R. C., FISCHINGER, P. J., BOLOGNESI, D. P.: Restricted neutralization of divergent human T-lymphotropic virus type-3 isolates by antibodies to the major envelope glycoprotein. Proc. Natl. Acad. Sci. U. S. A. **83**, 9707-9713 (1986).

104. McCULLOUGH, K. C.: Monoclonal antibodies: Implications for virology. Arch. Virol. **87**, 1-36 (1986).

105. McDOUGAL, J. S., KENNEDY, M. S., SLIGH, J. M., CORT, S. P., MAWLE, A., NICHOLSON, J. K. A.: Binding of HTLV-3/LAV to $T4^+$ T-cells by a complex of the 110kD viral protein and the T4 molecule. Science **231**, 382-385 (1986).

106. McGUIRE, T. C., CRAWFORD, T. B., HENSON, J. B.: Immunofluorescent localization of equine infectious anemia virus in tissues. Ame. J. Path. **62**, 283-292 (1971).

107. McGUIRE, T. C., HENSON, J.: Equine infectious anemia: Pathogenesis of persistent viral infection. Perspectives in Virology **8**, 229-247 (1973).

108. McGUIRE, T. C., CRAWFORD, T. B.: Immunology of a persistent

retrovirus infection: Equine infectious anemia. Adv. Vet. Sci. Comp. Med. **23**, 137-159 (1979).

109. MONTAGNIER, L., DAUGUET,C., AXLER, C., CHAMARET, S., NUGEYRE, M.T., REY, F., BARRE-SINOUSSI, F., CHERMANN, J.C.: A new type of retrovirus isolated from patients presenting with lymphandenopathy and acquired immune deficiency syndrome: structural and antigenic relatedness with equine infectious anemia virus. Annales de Virologie **135E**, 119-134 (1984).

110. MONTAGNIER, L., GRUEST, J., CHAMARET, S., DAGUET, C., AXLER, C., GUETARD, D., NUGEYRE, M. T., BARRE-SINOUSSI, F., CHERMANN, J. C., BRUNET, J. B., KLATZMANN, D., GLUCKMAN, J. C.: Adaptation of lymphadenopathy virus (LAV) to replication in EBV-transformed B lymphoblastoid cell lines. Science **225**, 63-66 (1984).

111. MONTELARO, R.C., BOLOGNESI, D. P.: Structure and morphogenesis of type-C retroviruses. Adv. Cancer Res. **28**, 63-69 (1978).

112. MONTELARO, R. C., LOHREY, N., PAREKH, B., BLAKENEY, E. W., ISSEL, C.J.: Isolation and comparative biochemical properties of the major internal polypeptides of equine infectious anemia virus. J. Virol. **42**, 1029-1038 (9182).

113. MONTELARO, R. C., PAREKH, B., ORREGO, A., ISSEL, C. J.: Antigenic variation during persistent infection by equine infectious anemia virus, a retrovirus. J. Biol. Chem. **259**, 10539-10544 (1984).

114. NAKAJIMA, H.: Physicochemical and biological characterization of equine infectious anemia virus. pp. 162-174. In Proc. 3rd Int. Conf. Equine Infectious Dis., Paris (1972) (Karger, Basel 1973)

115. NARAYAN, O., GRIFFIN, D. E., CHASE, J.: Antigenic drift of visna virus in persistently infected sheep. Science **197**, 376-378 (1977).

116. NARAYAN, O., WOLINSKY, J. A., CLEMENTS, J. E., STANDBERG, J. D., GRIFFIN, D. E., CORK, L. C.: Slow virus replication: The role of macrophages in the persistence and expression of visna viruses of sheep and goats. J. Gen. Virol. **59**, 345-356 (1982).

117. NARAYAN, O., KENNEDY-STOSKOPF, S., SHEFFER, D., GRIFFIN, D. E., CLEMENTS, J. E.: Activation of caprine arthritis-encephalitis virus expression during maturation of monocytes to macrophages. Infect. Immun. 41, 67-73 (1983).

118. NARAYAN. O., SHEFFER, D., CLEMENTS, J. E., TENNEKOON, G. Restricted replication of lentiviruses: Visna virus induce a unique interferon during interaction between lymphocytes and infected macrophages. J. Exp. Med. **162**, 1954-1969 (1985).

119. NICHOLSON, J. K. A., CROSS, G. D., CALLAWAY, C. S., McDOUGAL, J. S.: In vitro infection of human monocytes with human T lymphotropic virus type 3/lymphadenopathy-associated virus (HTLV-3/LAV). J. Immunol. **137**, 323-329 (1986).

120. NORRBY, E.: Immunogenecity of vaccine products and neutralizing antibodies. pp. 346-353. In Concepts in Viral Pathogenesis vol 2. Springer-Verlag, NY (1986).

121. NUMBERG, J. H., RODGERS, G., GILBERT, J. H., SNEAD, R. M. Method to map antigenic determinants recognized by monoclonal antibodies: Localization of a determinant of virus neutralization on the feline leukemia virus envelope protein gp-70. Proc. Natl. Acad. Sci. U.S.A **81**, 3675 (1984).

122. ORREGO, A., ISSEL, C.J., MONTELARO, R.C., ADAMS, W.V. Jr.: Virulence and in vitro growth of a cell adapted strain of equine infectious anemia virus after serial passage in ponies. Ame. J. Vet. Res. **43**, 1556-1560 (1982).

123. ORREGO, A.: Equine infectious anemia virus. 1. Generation of virus variant candidates. 2. Attempts to titrate by three different methods. Ph. D. dissertation, Louisiana State University, Baton Rouge, LA (1983).

124. PALESE, P.: Reassortment continuum. In Concepts in viral pathogenesis. In Concepts in viral pathogenesis. Edited by Abner Louis Notkins and Michael B.A. Oldstone. Springer-Verlag New York Inc. pp. 144-151 (1984).

125. PAREKH, B., ISSEL, C. J., MONTELARO, R. C.: Equine infectious anemia virus, a putative lentivirus contains polypeptides analogous to prototype-C oncrnaviruses. Virology **107**, 520-525 (1980).

126. PAYNE, S. L., PAREKH, B., MONTELARO, R. C., ISSEL, C. J.: Genomic alterations associated with persistent infections by equine infectious anemia virus, a retrovirus. J. Gen. Virol. **65**, 1395-1399 (1984).

127. PAYNE, S. L., SALINOVICH, O., NAUMAN, S. M., ISSEL, C. J., MONTELARO, R. C.: Course and extent of variation of equine infectious anemia virus during parallel persistent infections. J. Virol. **61**, 1266-1270 (1987).

128. PELUSO, R., HAASE, A., STROWRING, L.: A Trojan horse mechanism for the spread of visna virus in monocytes. Virology **147**, 231-236 (1985).

129. POPOVIC, M., SARGADHARAN, M. G., READ, E., GALLO, R. C.: Detection, isolation and continuous production of cytopathic retroviruses (HTLV-3) from patients with AIDS and pre-AIDS. Science **244**, 497-500 (1984).

130. POSSEE, R. D., SCHILD,G. C., DIMMOCK, N. J.: Studies on the mechanism of neutralization of influenza virus by antibody: evidence that neutralizing antibody (anti-haemagglutinin) inactivates influenza virus in vivo by inhibiting virion transcriptase activity. J. Gen. Virol. **58**, 378-386 (1982).

131. RATNER, L., HASELTINE, R., LIVAK, P. K., STARCICH, B., JOSEPHS, S., DORAN, R. E., RAFALSKI, J. A., WHITEHORN, E. A., BAUMEISTER, K., IVANOFF, L., PETTEWAY, S. R., LAUTENBERGER, I. A., PAPAS, T. S., GHRAYEB, J., CHANG, J., GALLO, R. C., WONG-STAAL, F.: Complete nucleotide sequence of the AIDS virus, HTLV-3. Nature **313**, 277-284 (1985).

132. REANNEY, D. C.: The evolution of RNA viruses. Annual Rev. Microbiol. **36**, 47-73 (1982).

133. RICE, N. R., SIMEK, S., RYDER, O. A., COGGINS, L.: Detection of proviral DNA in horse cells infected with equine infectious anemia virus. Journal of Virology **26**, 577-583 (1978).

134. ROGERS, G. N., PAULSON, J. C., DANIELS, R. S., SKEHEL, J. J., WILSON, I. A., WILEY, D. C.: Single amino acid substitutions in influenza hemagglutinin change receptor bindning specificity. Nature **340**, 76-78 (1983).

135. RUSHLOW, K., OLSEN, K., STIEGLER, G., PAYNE, S. L., MONTELARO, R. C., ISSEL, C. J.: Lentivirus genomic organization:

The complete nucleotide sequence of the env gene of equine infectious anemia virus. Virology **155**, 309-321 (1986).

136. SALINOVICH, O., PAYNE, S.L., MONTELARO, R. C., HUSSAIN, K. A., ISSEL, C. J., SCHNORR, K. L.: Rapid emergence of novel antigenic and genomic variants of equine infectious anemia virus during persistent infection. J. Virol. **57**, 71-80 (1986).

137. SCHNEIDER, J., KAADEN, O., COPELAND, T. D., OROSZLAN, S., HUNSMANN, G.: Shedding and interspecies type sero-reactivity of the envelope glycoprotein gp-120 of the human immunodeficiency virus. J. Gen. Virol. **67**, 2533-2538 (1986).

138. SCOTT, J. V., STOWRING, L., HAASE, A., NARAYAN, O., VIGNE, R.: Antigenic variation in visna virus. Cell **18**, 321-327 (1979).

139. SENTSUI, H., KONO, Y.: Hemagglutination by equine infectious anemia virus. Infect. Immun. **14**, 325-331 (1976).

140. SHANE, B. S., ISSEL, C. J., MONTELARO, R. C.: Enzyme-linked immunosorbent assay for the detection of equine infectious anemia p26 antigen and antibody. J. Gen. Microbiol. **19**, 351-355 (1984).

141. SHARPE, A. H., FIELDS, B. N.: Pathogenesis of viral infections. Basic concepts derived from the reovirus model. New Eng. J. Med. **312**, 486-497 (1985).

142. SHAW, G. M., HAHN, B. H., ARYA, S. K., GROOPMAN, J. E., GALLO, R.C., WONG-STAAL, F.: Molecular characterization of human T-cell leukemia (lymphotropic) virus type 3 in the acquired immune deficiency syndrome. Science **226**, 1165-1171 (1984).

143. SHAW, G. M., HARPER, M. E., HAHN, B. H., EPSTEIN, L. G., GAJ-DUSEK, D. C., PRICE, R. W., NAVIA, B. A., PETITO, C. K., O'HA-

RA,C. J.,GROOPMAN, J. E., CHO, E. S., OLESKE, J. M., WONG-STAAL, F., GALLO, R. C.: HTLV-3 infection in brains of children and adults with AIDS encephalopathy. Science 227, 177-182 (1984).

144. SHEN, D., CRAWFORD, T. B., GORHAM, J. R., McGUIRE, T. C.: Inactivation of equine infectious anemia virus by chemical disinfectants. Ame. J. Vet..Res. **38(8)**, 1217-1219 (1977).

145. SHEN, D. T., GORHAM, J. R., JONES, R. H., CRAWFORD, T. B.: Failure to propagate equine infectious anemia virus in mosquitoes and Culicoides variipennis. Ame. J. Vet. Res. **39**, 875-876 (1978).

146. SKEHEL, J. J., DANIELS, D. S., DOUGLAS, A. R., WILEY, D. C.: Antigenic and amino acid sequence variations in the hemagglutinin of type A influenza viruses recently isolated from human subjects. Bullettin W.H.O. **61**, 671-676 (1983).

147. STARCICH, B., HAHN, B. H., SHAW, G. M., MCNEELY, P., MODROW, S., WOLF, H., PARKS, E. S., PARKS, W. P., JOSEPHS, S., GALLO, R. C., WONG-STAAL, F.: Identification and characterization of conserved and variable regions in the envelope gene of HTLV/LAV, the retrovirus of AIDS. Cell **45**, 637-648 (1986).

148. STEPHENS, R. M., CASEY, J. W., RICE, N. R.: Equine infectious anemia virus gag and pol genes: Relatedness to visna virus and AIDS virus. Science **231**, 589-594 (1986).

149. STROOP, W. G., BARINGER, J. R. Persistent, slow and latent viral infections. Prog. Med. Virol. **28**, 1-43 (1982).

150. TARDIEU, M., EPSTEIN, R. L., WEINER, H. L.: Interaction of viruses with cell surface receptors. Int. Rev. Cytol. **80**, 27-61 (1982).

151. TAYLOR, H. P., DIMMOCK, N. J.: Mechanisms of neutralization of influenza virus by IgM. J. Gen. Virol. **66**, 903-907 (1985).

152. TAYLOR, H. P., DIMMOCK, N. J.: Mechanisms of neutralization of influenza virus by secretory IgA is different from that of monomeric IgA or IgG. J. Exp. Med. **161**, 198-209 (1985).

153. THORMAR, H., BARSHATZKY, M. R., KOZLOWSKI, P. B.: The emergence of antigenic variation ia a rare event in long term visna virus infection in vivo. J. Gen. Virol. **64**, 1427-1432 (1983).

154. TEICH, N.: Taxanomy of retroviruses. pp. 25-207. In Molecular Biology of Tumor Viruses: RNA Tumor viruses. 2nd Edition, Weiss, R., Teich, N., Varmus, H., and Coffin, J. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984).

155. VARMUS, H., SWANSTROM, R.: Replication of retroviruses. pp. 369-512. In Molecular Biology of Tumor Viruses: RNA Tumor viruses. 2nd Edition, Weiss, R., Teich, N., Varmus, H., and Coffin, J. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984).

156. VIRELIZIER, J. L.: Host defenses against influenza virus: the role of anti-hemagglutinin antibody. J. Immunol. **115**, 434-439 (1975).

157. WEBSTER, R. G., LAVER, W. G.: Determination of the number of non-overlapping antigenic areas on HongKong (H3N2) influenza virus hemagglutinin with monoclonal antibodies and the selection of variants with potential epidemiological significance. Virology **104**, 139-148 (1980).

158. WEILAND, F., MATHEKA, H., COGGINS, L., HARTNER, D.: Electron microscopic studies on equine infectious anemia virus (EIAV). Arch. Virol. **55**, 335-340.

159. WEISS R. A., CLAPHAM, P. R., CHEINGSONG-POPOV, R., DALGLEISH, A. G., CARNE, C. A., WELLER, I. V. D., TEDDER, R. S.: Neutralization of HTLV-3 by sera of AIDS and AIDS-risk patients. Nature **316**, 69-72 (1985).

160. WEISS, R. A., CLAPMAN, P. R., WEBER, J. N., DALGLEISH, A. G., LASKY, L. A., BERMAN, P. W.: Variable- and conserved neutralization antigens of human immunodeficiency virus. Nature **324**, 572-575 (1986).

161. WIKTOR, J. J., MACFARLAN, R. I., REAGAN, K. J., DIETZSCHOLD, B., CURTIS, P. J., WUNNER, W. H., KIENY, M. P., LATHE, R., LECOQ, J. P., MACKETT, M., MOSS, B., KOPROWSKI, H.: Protection from rabies virus by a vaccinia virus recombinant containing the rabies virus glycoprotein gene. Proc. Natl. Acad. Sci. U.S.A **81**, 7194 (1984).

162. WILLEY, R. L., RUTLEDGE, R. A., DIASS, S., FOLKS, T., THEODORE, T., BUCKLER, C. E., MARTIN, M. A.: Identification of conserved and divergent domains within the envelope gene of the cquired immunodeficiency syndrome retrovirus. Proc. Natl. Acad. Sci. U.S.A. **83**, 5038-5042 (1986).

163. WONG-STAAL, F., SHAW, G. M., HAHN, B. H., SALAHUDDIN, S. Z., POPOVIC, M., MARKHAM, P., REDFIELD, R., GALLO, R. C.: Genomic diversity of human T-lymphotropic virus type 3 (HTLV-3). Science **229**, 759-762 (1985).

164. YANIV, A., DAHLBERG, J. E., TRONICK, S. R., CHIU, I. M., AARONSON, S.A.: Molecular cloning of integrated caprine arthritis-encephalitis virus. Virology **145**, 340-345 (1985).

165. YANIV, A., DAHLBERG, J. E., GAZIT, A., SHERMAN, L., TRONICK, S.R., CHIU, I. M., AARONSON, S. A.: Molecular cloning and physical characterization of integrated equine infectious anemia virus: Molecular and immunologic evidence of its close relationship to ovine and caprine lentiviruses. Virology **154**, 1-8 (1986).

166. YEWDELL, J. W., BENNINK, J. R., SMITH, G. L., MOSS, B.: Influenza A virus nucleoprotein is a major target antigen for cross-reactive anti-influenza A virus cytotoxic T lymphocytes. Proc. Natl. Acad. Sci. U. S. A. 82, 1785-1789 (1986).

167. YEWDELL, J. W., CATON, A. J., GERHARD, W.: Selection of influenza A virus adsorptive mutants by growth in the presence of a mixture of monoclonal antihemagglutinin antibodies. J. Virol. **57**, 623-628 (1986).

CHAPTER TWO

EQUINE INFECTIOUS ANEMIA VIRUS (EIAV): HUMORAL RESPONSES AND ANTIGENIC VARIATION DURING PERSISTENT INFECTION IN PONIES.

SUMMARY

Three Shetland ponies (A, B and C) were inoculated with plasma from a pony with clinical signs of equine infectious anemia (EIA) and observed for 165 to 440 days. Clinical signs of EIA marked by high fever (39.5^{0}C) lasting 2-4 days were observed 2-3 weeks after infection and one to four such episodes occurred in each pony. EIAV was reisolated from the plasma of each pony by end-point dilution in fetal equine kidney (FEK) cells and a plasma viremia $10^{3.5}$ $TCID_{50}$/ml was observed during each episode. Cross-neutralization tests with sequential serum samples showed that the four viruses isolated from pony A during febrile episodes 4-5 weeks apart were serologically different. Two-dimensional tryptic peptide analyses of the envelope glycoproteins of four isolates from pony A revealed additions or deletions between each isolate and its immediate predecessor. Western blot analyses of these four isolates including those from ponies B and C with a panel of monoclonal antibodies demonstrated antigenic variation in both gp90 and gp45. Both conserved and variable epitopes were identified in each glycoprotein with greatest variation in gp90. Specific IgG directed against determinants of EIAV gp90, gp45 and p26 was detectable by AGID and Western blots within one month after infection. These antibodies appeared to react with conserved antigenic

determinants of these proteins as they reacted with homologous and heterologous virus isolates alike. The group-specific determinants of gp90 and gp45 were more antigenic than those of p26. The ability of "early" sera to recognize determinants on all EIAV isolates did not correlate with their ability to neutralize the infectivity of EIAV. Neutralizing antibodies, first detectable within two months, were observed to be effective only to viruses isolated prior to serum collection but became broadly reactive later in the infection regardless of the number of clinical episodes.

INTRODUCTION

Equine infectious anemia virus (EIAV) a lentivirus that shares morphological, serological and genetic similarity with human immunodeficiency virus (HIV) (8, 19, 33, 34) causes a lifelong persistent infection in horses often characterized by recurrent episodes of high fever (3, 12). Major virion envelope glycoproteins present the principal targets for immune attack against retroviruses (32). Monoclonal antibodies to some epitopes of the major envelope glycoprotein of EIAV, gp90, have been shown to neutralize virus infectivity (11). In addition to their ability as retroviruses to persist in their hosts as integrated proviruses, the lentiviruses further elude protective immune responses because the antigenic structures of the surface glycoproteins change (11, 22, 30, 31). Biochemical analyses of the env gene of the lentiviruses have demonstrated marked variability particularly in the region coding for the outer envelope protein (4, 9, 10, 27, 28, 35). The rapid antigenic variation common in the lentiviruses suggests that development of vaccines for these viruses will be difficult. In EIAV, where genomic variations often result in the emergence of antigenic variants that cause recurrent febrile episodes (16, 26, 27, 28), the extent of antigenic variation needs to be determined with the hope that suitable immunogens can be identified.

To extend our previous studies on the antigenic variation of EIAV, seven virus isolates recovered from persistently infected ponies were analyzed by cross-neutralization with convalescent sera. To determine the changes that occur in EIAV proteins during antigenic variation, the

envelope glycoproteins of the virus isolates were analyzed in two-dimensional tryptic peptide maps, Western blots with homologous and heterologous equine sera, and a panel 18 monoclonal antibodies. The results document the generation of group- and type-specific antibodies to EIAV glycoproteins and extend our previous data indicating that during persistent infections the virus undergoes rapid antigenic variation by altering the structure of the envelope glycoproteins.

MATERIALS AND METHODS

Animals and viral challenge: Serial passage of the prototype cell adapted Wyoming strain of EIAV (18) in Shetland ponies and the isolation of virus from plasma has been previously described (23). In this study, plasma obtained during the first febrile episode of a second passage recipient pony (#82) was used as source of challenge virus (23). This plasma has been the source of several well characterized antigenic variants of EIAV (11, 27, 28, 31). Briefly, three Shetland ponies F135, P135 and P130 (subsequently referred to as A, B, and C, respectively) were each inoculated intravenously with approximately $10^{4.8}$ median tissue culture infective doses ($TCID_{50}$) of EIAV in plasma. Rectal temperatures were daily recorded for a period ranging from 165 to 440 days post inoculation (DPI). Plasma samples obtained during febrile episodes ($39.5^{0}C$) (Figure 1) were the source of EIAV isolates used in this study.

Virus isolation, production and purification: Isolation of virus from plasma and propagation were performed in fetal equine kidney (FEK) cell

cultures using previously described methods (20, 23). The predominant virus in each febrile episode was isolated by end-point dilution in FEK cell cultures. Production of EIAV antigen in these cells was monitored in an agar gel immunodiffusion (AGID) test (1). End-point diluted viruses were produced in FEK cell cultures and purified on 20-80% glycerol gradients according to previously described methods (20, 24).

Equine convalescent sera: Sera collected from experimentally infected ponies and naturally infected horses were used to characterize virus isolates in neutralization and immunoblot assays. Sera from experimentally infected ponies were collected between febrile episodes and during the afebrile periods. Sera from naturally infected horses were selected from horses known to be infected (by AGID test seropositivity) for over 10 years.

Detection of antibodies to EIAV: Antibodies to EIAV were detected in the agar gel immunodiffusion (AGID) test using approved protocols (29) and commercially available reagents (Pittman-Moore, Washington Crossing, NJ), in virus neutralization and in immunoblot assays.

Virus neutralization assay: The neutralizing activities of convalescent sera were determined in FEK cell cultures using the alpha-method of virus neutralization. Viruses isolated from plasma were reacted with serum collected both before and after each febrile episode. Serial 10-fold dilutions of each virus were mixed with a standard dilution (1:4 in MEM) of heat inactivated (56^0C, 1 h) serum. The virus-serum mixtures were

incubated at 37°C for 1 h and assayed for residual infectious virus by inoculating duplicate FEK cell monolayers with 1 ml aliquots. Controls consisting of mixtures of virus dilutions with MEM were included for each test. For virus adsorption, inoculated cultures were incubated at 37°C for 1 h. Maintenance medium (5 ml) was added to each culture and incubation at 37°C was continued for 3 weeks when cultures were assayed for virus antigens according to previously described methods (1). The neutralization index for each serum was calculated as the difference in virus titre without serum and that of the virus-serum mixture. A neutralization index of 1.7 was considered positive.

Monoclonal antibodies: The isolation and characterization of a panel of monoclonal antibodies (MAbs) to glycoproteins of prototype EIAV has previously been described (11). These and other MAbs were used to determine the antigenic changes in seven EIAV isolates. Two-month old BALB/c mice were immunized with purified EIAV isolate A/1 using previously described protocols (11). Production of anti-virus antibodies was monitored in an indirect enzyme-linked immunosorbent assay (ELISA). Mice were sacrificed 3 days after the final boost and their spleen cells fused with SP2/0 myeloma cells in the presence of PEG (50% PEG in RPMI-1640 medium) (7). Hybridomas were screened for specific antibody production in ELISA (11) and Western blots and those secreting viral glycoprotein specific antibodies were subcloned twice by limiting dilution. Ascites was obtained in pristane primed mice as previously described (11).

Immunoblot analysis of EIAV isolates with equine sera and MAbs: EIAV proteins were separated on 7.5 to 20% gradient gels in discontinuous buffer under reducing conditions (17) and blotted onto nitrocellulose membranes using previously described methods (2, 13). The nitrocellulose membranes were blocked for 2 h with 5% BLOTTO in TBS (10mM Tris-HCL, 0.9% NaCl, pH 7.4) to prevent non-specific binding of antibody (14) and incubated with MAbs (1:20 dilution of ascites or 1:4 dilution of tissue culture fluid) or equine serum (at indicated dilutions) for 2 h. All incubations with antibody or with TBS containing 0.05% Tween 20 (SIGMA Chemicals, St. Louis, MO.) were done at 37^0C with agitation. After a 20 minute wash in TBS/Tween 20 the membranes were incubated for 1 h with horseradish peroxidase labeled second antibody. Affinity purified goat anti-mouse immunoglobulins (CAPPEL, COOPER BIOMEDICAL INC., MALVERN, PA) or rabbit anti-horse IgG (MILES-YEDA LTD, ISRAEL) were used at a dilution of 1:1000 or 1:3000, respectively. After two washes (final wash with TBS) bands specifically recognized by equine sera or monoclonal antibodies were developed with 4-chloro-1-naphthol in methanol (Bio-Rad, Richmond, CA).

Glycoprotein purification and peptide mapping: EIAV glycoproteins (gp90 and gp45) from isolates A/1-4 were purified on lentil-lectin affinity columns as previously described (21). Affinity purified glycoproteins were separated on discontinuous 10% SDS-PAGE (17). Gel slices containing gp90 and gp45 were radioiodinated and digested with trypsin. The released radioiodinated peptides were analyzed by two-dimensional thin layer chromatography and detected by autoradiography with Kodak X-

Omat film (XRP-5) (5, 6, 22, 31).

RESULTS

Persistent infection in Shetland ponies.

First febrile episodes (defined as 39.5^{0}C for 2 or more days) were observed between 16 and 19 DPI (Fig 1). Antibodies to EIAV antigens were detected in AGID on 25 DPI (A and B) and 29 DPI (C). After the first febrile episode the clinical course of the disease was variable. Ponies A and B had four and two febrile episodes, respectively, and pony C had only one febrile episode (Fig. 1). The duration of the febrile episodes ranged from 2 to 4 days and each episode was characterized by high fever, depression, inappetence and general weakness. All the episodes occurred within four months of infection. Ponies A and B died on 280 and 165 DPI, respectively, after several days of severe illness marked by depression, anorexia, loss of weight, prostration and diarrhoea. Pony C was euthanatized on 440 DPI after a similar severe illness.

Plasma samples collected during febrile episodes contained from $10^{3.5}$ to $10^{4.5}$ $TCID_{50}$ of EIAV per ml (Fig 1). A total of seven isolates of EIAV were recovered from plasma by end-point dilution in FEK cell cultures; virus isolates from sequential episodes from the same pony are numbered sequentially, e.g. A/1, A/2 etc. (Fig 1). The serologic characteristics of these viruses were analyzed in neutralization assays with convalescent sera, immunoblots with homologous and heterologous convalescent sera and MAbs. The structural alterations of EIAV glycoproteins were analyzed by tryptic peptide maps.

Immunoblot analysis of equine sera during persistent infection with EIAV.

The protein binding specificities of sera collected during persistent infection with EIAV were analyzed in immunoblot assay. To obtain maximum transfer of gp90 and gp45, electroblotting from SDS-PAGE gels to nitrocellulose membranes was continued for 22 h at 75 V. With shorter transfer times, immunoreactivity with gp90 was less intense. Preinoculation sera were negative for EIAV specific antibodies (Fig. 2 panel A). Serum collected after the first febrile episode from pony A at 35 DPI had detectable activity to EIAV gp90, gp45 and p26 of isolates A/1, A/4 and prototype EIAV (Fig 2 panel B). The predominant activity was to the major envelope glycoprotein gp90 and the major core protein p26 at the 1:50 dilution used (Fig 2 panel B). The serum had similar activity with the other 5 isolates. Comparison of the seven isolates with prototype EIAV in SDS-PAGE showed minor variation in the rate of migration of the envelope glycoproteins with no variation in the migration of the core proteins (data not shown). Serum collected at 203 DPI contained high levels of antibody that bound extensively with gp90, gp45 and p26 (Fig. 2 panel C). Several other bands were noted at the 1:100 dilution used. As the level of IgG to these minor bands was generally low in infected horses, only those bands corresponding to gp90, gp45 and p26 were considered in the evaluation of humoral responses to EIAV isolates. Sera from naturally infected horses reacted with determinants of gp90, gp45 and p26 in the same viruses with levels of p26 activity lower than those to the envelope glycoproteins in most samples (Fig. 2

panel D, and Fig. 3). Immunoblot analysis using serial dilutions of both 35 DPI and 203 DPI sera indicated that the envelope glycoproteins are more highly immunogenic relative to their mass in the mature virion (Table 1).

Immunoblot analysis of EIAV isolates with MAbs to gp90 and gp45.

The antigenic alterations in gp90 and gp45 during persistent infection were analyzed with MAbs. The activity of MAbs to gp90 (F1-120 and F1-123) and gp45 (F1-106, F1-102 and F1-110) of isolate A/1 was tested against seven virus isolates including the prototype cell adapted EIAV (Table 2). The activity of these MAbs was analogous to that of MAbs previously shown to recognize epitopes 90-A and 45-A, respectively (11). As these MAbs appeared to be directed against highly conserved epitopes of EIAV they were grouped with those mapping to epitopes 90-A and 45-A, respectively. Analysis of viruses isolated from sequential and parallel febrile episodes with MAbs to prototype EIAV demonstrated the presence of variable and conserved epitopes in gp90 and gp45, respectively (Table 3). The previously described classification of epitopes in gp90 (A-F) and gp45 (A-B) (11) was used in this study. Epitope 90-E was not detected in blots with all the seven virus isolates. The greatest antigenic variation was observed in the major envelope glycoprotein, gp90.

Appearance and specificity of EIAV neutralizing antibody.

Neutralizing activity was present in all three ponies by 67 DPI and was effective only for homologous virus isolates recovered prior to

collection of the serum, i.e. 16 or 46 DPI (Table 4). Cross-neutralization with sequentially collected sera showed that isolates A/1-4 were serologically different. Serum collected from pony A later in the course of infection, i.e. at 203 DPI, had neutralizing activity for all virus isolates recovered earlier in the infection (Table 4). Similar neutralizing activity was observed with sera collected from pony B. Serum collected from pony C late in the infection (438 DPI) neutralized several isolates of EIAV including the prototype virus (Table 5). The neutralization activity of 438 DPI serum was broad but could still distinguish serological differences between the panel of virus isolates tested (Table 5). The specificity of neutralizing antibodies in late serum was broad and did not correlate with the number of sequential febrile episodes observed.

Analysis by peptide mapping.

The structures of the envelope glycoproteins of four virus isolates recovered from sequential febrile episodes were analyzed by two-dimensional tryptic peptide mapping procedures. The peptide maps of the gp90 components of isolates A/1-A/4 (Fig. 4 row A) indicate peptide additions and deletions when each virus isolate is compared with its most immediate progenitor. Thus, A/2 is compared to A/1, A/3 to A/2 and A/4 to A/3, respectively. The results of each comparison revealed differences in the peptide maps of each pair examined. Between any two pairs examined, isolate A/4 had the greatest number of peptide additions. Peptide maps were also obtained for the respective gp45 components of isolates A/1-A/4 (Fig 4 row B). Again pair-wise comparisons of the gp45 peptide maps revealed the presence of additions and deletions.

DISCUSSION

Our data on the antigenic variation of EIAV are in agreement with previous reports (11, 16, 22, 27, 28, 31). We further show in this report the kinetics of humoral responses to EIAV during experimental infections and extend our previous data on the antigenic and structural alterations occurring in the envelope glycoproteins during persistent infections.

In this study, the decline of viremia did not correlate with detection of antibodies capable of neutralizing EIAV in equine fibroblast cell cultures. Neutralizing activity was detectable within two months and was effective against only those viruses recovered during prior febrile episodes suggesting that the neutralizing antibodies were type specific. The occurrence of recurrent febrile episodes and the specificity of neutralizing antibodies observed during chronic EIA suggest that conserved antigenic determinants detectable in the envelope glycoproteins do not elicit sufficient protective immune responses. The specificity of neutralizing antibody observed in vitro broadened during the course of EIA suggesting that multiple stimulation of the immune system with one or more EIAV strains was necessary to generate this activity. These data are in agreement with those of Kono et al. (15) who showed that neutralizing antibody as assayed in horse leukocyte cultures appeared from 45 to 87 DPI and reached peak titers from 90 to 148 days. The rapid appearance of high levels of neutralizing antibodies to the isolates recovered after 45 DPI is suggestive of either immunological priming with heterologous isolates or the presence of sensitizing levels of homologous isolates preceding each recurrent febrile episode. These studies showed that the neutralizing activity persisted for a long time. Although all

three ponies died within one and half years after infection despite the presence of potent neutralizing antibodies, several ponies infected with the plasma used in this study have lived for over 8 years suggesting that early death may have been due to other causes.

The envelope glycoproteins of prototype EIAV and the seven isolates in this study contain highly antigenic group-specific determinants which in immunoblot assays reacted strongly and more consistently than those associated with the major core protein, p26. This and other studies (13) have shown that in many naturally infected horses, antibodies to the viral core proteins are often undetectable in Western blots when serum is diluted 1:50. In contrast, activity with the envelope glycoproteins was uniformly detected in sera with positive AGID results. No correlation was detected between the length of infection and the levels of antibodies to the core proteins. The lower levels of antibodies to p26 could result from abundant production of core proteins that may remove specific antibody from circulation.

We have previously demonstrated that virus isolates recovered from ponies inoculated with the virus strain used in this study could be distinguished with panels of MAbs to gp90 and gp45 of EIAV (11). Both conserved and variable epitopes have been identified in gp90 and gp45, respectively (11). Analysis of seven isolates with the same panel of MAbs demonstrated antigenic variability in gp90 and gp45. Most of the epitopes in gp90 were variable and appeared to evolve randomly. The antigenic variation occurred both before and after the appearance of detectable neutralizing antibodies suggesting that selection processes other than by neutralizing antibodies are involved in EIA. Similar observations have

been made in other studies which demonstrated nucleotide, oligonucleotide, peptide and glycopeptide variation in isolates recovered from febrile episodes that occurred 2-3 weeks after infection (22, 27, 28, 31). The random appearance and disappearance of epitopes in the major envelope glycoprotein and the pattern of neutralization observed with convalescent sera suggest that the neutralization antigenic sites are altered during persistent infection.

Tryptic peptide mapping of gp90 and gp45 components of four virus isolates (A/1-A/4) recovered from sequential episodes separated by as little as 4 weeks showed several peptide map additions and deletions occurring between each isolate and its predecessor. Our data are in agreement with results of previous analyses of EIAV isolates and further indicate that each febrile episode is caused by a distinct virus population (22, 31). Cross-neutralization assays, Western blotting with MAbs and peptide mapping of EIAV isolates indicate that each procedure may classify the variants into different groups. The maps generated for these virus isolates were different from those previously reported for parallel isolates (22, 31). Previous studies have shown alterations in the peptide, glycopeptide and oligonucleotide maps of isolates recovered during sequential febrile episodes (26, 28, 31). More recently we have shown that isolates with altered immunoreactivity patterns, peptide, glycopeptide or oligonucleotide maps have numerous clustered base substitutions in the region coding for the outer envelope glycoprotein indicating that the observed antigenic and structural variations have a genetic basis (28).

Antigenic variation in this virus can therefore be explained by the appearance of random point mutations in the envelope gene. Mutations

affecting amino acid residues that either form neutralization antigenic sites or which are crucial in the tertiary folding of the envelope glycoproteins can alter the antigenicity of the protein and render it unreactive with existing protective immune factors. Moreover, alteration of glycosylation patterns may alter the antigenicity of the viral glycoproteins allowing progeny virus to infect cells in the presence of immune factors specific for parental virus. The observation that cessation of recurrent febrile episodes was associated with presence of neutralizing antibodies with broad specificity suggests that multiple stimulations of the immune system with several strains of EIAV may generate the necessary immune factors important in the control of virus replication. Although the nature of these immune factors that eventually control the cycles of virus replication have not yet been identified, results lead us to suggest that immunogens derived from several strains or serotypes of EIAV may be needed to confer protection.

REFERENCES

1. AMBORSKI, G. F., JEFFERS, G., AMBORSKI, R. L., ISSEL, C. J.: Equine infectious anemia virus: Development of a simple reproducible method for titrating infectivity of the cell-adapted strain. Am. J. Vet. Res. **40**, 302-304 (1979).
2. BURNETTE, W. N.: "Western blotting" electrophoretic transfer of proteins from -sodium dodecylsulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112**, 195-203 (1981).
3. CHEEVERS, W. P., McGUIRE, T. C.: Equine Infectious Anemia Virus: Immunopathogenesis and persistence. Rev. Infect. Dis. **7(1)**, 83-88 (1985).
4. CLEMENTS, J. E., PEDERSON, F. S., NARAYAN, O., HASELTINE, W. A.: Genomic changes associated with antigenic variation of visna virus during persistent infection. Proc. Natl. Acad. Sci. USA **77**, 4454-4458 (1980).
5 ELDER, J., GAUTSCH, J. W., JENSEN, F. C., LERNER R. A. HARTLEY, J. W., ROWE, W. P.: Biochemical evidence that MCF murine leukemia viruses are envelope (env) gene recombinants. Proc. Natl. Acad. Sci. USA **74**, 4676-4680 (1977a).
6. ELDER, J., PICKET, R. 2., HAMPTON, J., LERNER, R.: Radioiodination of proteins in single polyacrylamide gel slices. J. Biol. Chem. **252**, 6510-6515 (1977b).
7. GALFRE, G. & MILSTEIN, C. (1981). Preparation of monoclonal antibodies: strategies and procedures. Methods in Enzymology **73**, 1-46.

8. GONDA, M. A., WONG-STAAL, F., GALLO, R. C., CLEMENTS, J. E., NARAYAN, O., GELDEN, R. V.: Sequence homology and morphologic similarity of HTLV-3 and visna virus, a pathogenic lentivirus. Science **227**, 173-177 (1985).

9. HAHN, B. H., GONDA, M. A., SHAW, G. M., POPOVIC, M., HOXIE, J. A., GALLO, R. C., WONG-STAAL, F.: Genomic diversity of the acquired immune deficiency syndrome virus HTLV-3: Different virus exhibit greatest divergence in their envelope genes. Proc. Natl. Acad. Sci. USA **82**, 4813-4817 (1985).

10. HAHN, B. H., SHAW, G. M., TAYLOR, M. E., REDFIELD, R. R., MARKHAM, P. D., SALAHUDDIN, S. Z., WONG-STAAL, F., GALLO, R. C., PARKS, E. S., PARKS, W. P.: Genetic variation in HTLV-3/LAV over time in patients with AIDS or at risk for AIDS. Science **232**, 1548-1554 (1986).

11. HUSSAIN, K. A., ISSEL, C. J., SCHNORR, K. L., RWAMBO, P. M., MONTELARO, R. C.: Antigenic analysis of equine infectious anemia virus (EIAV) variants using monoclonal antibodies: epitopes of glycoprotein 90 (gp90) of EIAV stimulate neutralizing antibodies. J. Virol. **61**, 2956-2961 (1987).

12. ISSEL, C.J., COGGINS, L.: Equine infectious anemia: Current knowledge. J. Am. Vet. Med. Assoc. **174**, 727-733 (1979).

13. ISSEL, C. J., RWAMBO, P. M., MONTELARO, R. C.: Evolution of equine infectious anemia diagnostic tests: Recognition of a need for detection of anti-EIAV glycoprotein antibodies. Proc. 5th Int. Conf. Eq. Infect. Dis., University of Kentucky Press, Lexington, KY. (in press).

14. JOHNSON, D. A., GAUTSCH, J. W., SPORTSMAN, J. R., ELDER, J. H.: Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Gene Anal. Techn. **1**, 3-8 (1984).

15. KONO, Y., KOBAYASHI, K., FUKUNAGA, Y.: Serological comparison among various strains of equine infectious anemia virus. Arch. Virol. **34**, 202-208 (1971).

16. KONO, Y., KOBAYASHI, K., FUKUNAGA, Y.: Antigenic drift of equine infectious anemia virus in chronically infected horses. Arch. Virol. **41**, 1-10 (1973).

17. LAEMMLI, U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**, 680-685 (1970).

18. MALMQUIST, W. A., BARNETTE, D., BECVAR, C. S.: Production of equine infectious anemia virus antigen in a persistently infected cell line. Arch. Virol. **42**, 361-370 (1973).

19. MONTAGNIER, L., DAUGUET, C., AXLER, C., CHAMARET, S., NUGEYRE, M.T., REY, F., BARRE-SINOUSSI, F., CHERMANN, J. C.: A new type of retrovirus isolated from patients presenting with lymphadenopathy and acquired immune deficiency syndrome: structural and antigenic relatedness with equine infectious anemia virus. Annales de Virologie (Institut Pasteur) **135E**, 119-134 (1984).

20. MONTELARO, R. C., LOHREY, N., PAREKH, B., BLAKENEY, E. W., ISSEL, C. J.: Isolation and comparative biochemical properties of the major internal polypeptides of equine infectious anemia virus. J. Virol. **42**, 1029-1038 (1982).

21. MONTELARO, R. C., WEST, M. ISSEL, C. J.: Isolation of equine

infectious anemia virus glycoproteins. Lectin affinity chromatography procedures for high avidity glycoproteins. J. Virol. Methods. **6**, 337-346 (1983).

22. MONTELARO, R. C., PAREKH, B., ORREGO, A., ISSEL, C. J.: Antigenic variation during persistent infection by equine infectious anemia virus, a retrovirus. J. Biol. Chem. **259**, 10539-10544 (1984).
23. ORREGO, A., ISSEL, C. J., MONTELARO, R. C., ADAMS, W. V. Jr.: Virulence and in vitro growth of a cell adapted strain of equine infectious anemia virus after serial passage in ponies. Am. J. Vet. Res. **43**, 1556-1560 (1982).
24. ORREGO, A.: Equine infectious anemia virus: (1) the generation of virus variant candidates by serial passage in Shetland ponies, (2) attempts to titrate the virus by three different methods. Ph. D. dissertation, Louisiana State University, (1983).
25. PAREKH, B., ISSEL, C. J., MONTELARO, R. C.: Equine infectious anemia virus, a putative lentivirus contains polypeptides analogous to prototype-C oncrnaviruses. Virology **107**, 520-525 (1980).
26. PAYNE, S. L., PAREKH, B., MONTELARO, R. C., ISSEL, C. J.: Genomic alterations associated with persistent infections by equine infectious anemia virus, a retrovirus. J. Gen. Virol. **65**, 1395-1399 (1984).
27. PAYNE, S. L., SALINOVICH, O., NAUMAN, S. M., ISSEL, C. J., MONTELARO, R. C.: Course and extent of variation of equine infectious anemia virus during parallel persistent infections. J. Virol. **61**, 1266-1270 (1987a).
28. PAYNE, S. L., FANG FU-DE, LIU CHENG-PIN, DHRUVA, B. R.,

RWAMBO, P., ISSEL, C. J., MONTELARO, R. C.: Antigenic variation and Lentivirus persistence: variations in envelope gene sequences during EIAV infection resemble changes reported for sequential isolates of HIV. Virology **161**, " in press".

29. PEARSON, J. E., COGGINS, L.: Protocol for the immunodiffusion (Coggins) test for equine infectious anemia. Proc. Am. Assoc. Vet. Lab. Diag. **18**, 1-13 (1979).
30. RICE, N. R., SIMEK, S., RYDER, O. A., COGGINS, L.: Detection of proviral DNA in horse cells infected with equine infectious anemia virus. J. Virol. **26**, 577-583 (1978).
31. SALINOVICH, O., PAYNE, S. L., MONTELARO, R. C., HUSSAIN, K. A., ISSEL, C. J., SCHNORR, K. L.: Rapid emergence of novel antigenic and genomic variants of equine infectious anemia virus during persistent infection. J. Virol. **57**, 71-80 (1986).
32. SCHAFER, W., BOLOGNESI, D. P.: Mammalian C-type oncornaviruses: relationships between viral structure and cell-surface antigens and their possible significance in immunological defense mechanisms. Contemp. Top. Immunobiol. **6**, 127-167 (1977).
33. SCHNEIDER, J., KAADEN, O., COPELAND, T. D., OROSZLAN, S., HUNSMANN, G.: Shedding and interspecies type sero-reactivity of the envelope glycoprotein gp-120 of the human immunodeficiency virus. J. Gen. Virol. **67**, 2533-2538 (1986).
34. STEPHENS, R. M., CASEY, J. W., RICE, N. R.: Equine infectious anemia virus gag and pol genes: Relatedness to visna virus and AIDS virus. Science **231**, 589-594 (1986).
35. WLLLEY, R. L., RUTLEDGE, R. A., DIAS, S., FOLKS, T., THEODORE,

T., BUCKLER, C. E., MARTIN, M. A.: Identification of conserved and divergent domains within the envelope gene of the acquired immunodeficiency syndrome retrovirus. Proc. Natl. Acad. Sci. U.S.A. **83**, 5038-5042 (1986).

Fig. 1: Clinical histories of Shetland ponies experimentally infected with EIAV. All sustained rectal temperature recordings 39.5^{0}C (dashed line) were considered febrile episodes. The infectivity titers in FEK cells of plasma obtained during the febrile periods are shown as $\log_{10}$ $TCID_{50}$/ml (vertical bars). Virus isolates recovered from the respective febrile episodes are shown, e.g. A/1 to A/4, etc. (A) pony F135, (B) pony P135 and (C) pony P130.

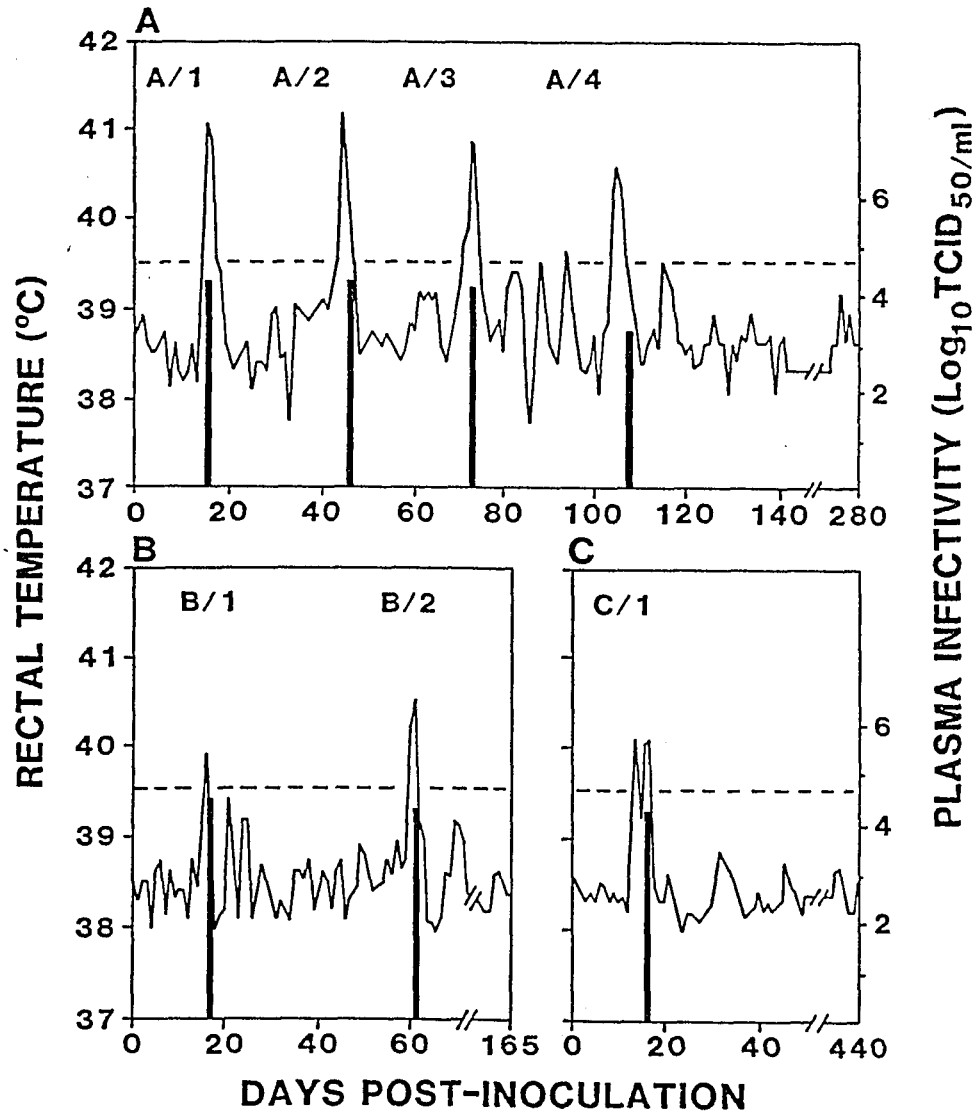

A
A/1
A/2
A/3
A/4
B
B/1
B/2
C
C/1
RECTAL TEMPERATURE (°C)
PLASMA INFECTIVITY (Log10TCID50/ml)
DAYS POST-INOCULATION

Fig. 2: Western blot reactivity patterns of convalescent sera during persistent infections. Representative data for the patterns of protein specificity of serum antibody are presented. Pre-inoculation serum at 1:50 dilution against isolate A/1 (panel A). Serum from pony A on 35 DPI (1:50) (panel B) and 203 DPI (1:100) (panel C), respectively and EIA reference serum (1:100) (panel D). Viruses A/1, A/4 and prototype EIAV are in lanes 1, 2 and 3 of panels B-D, respectively.

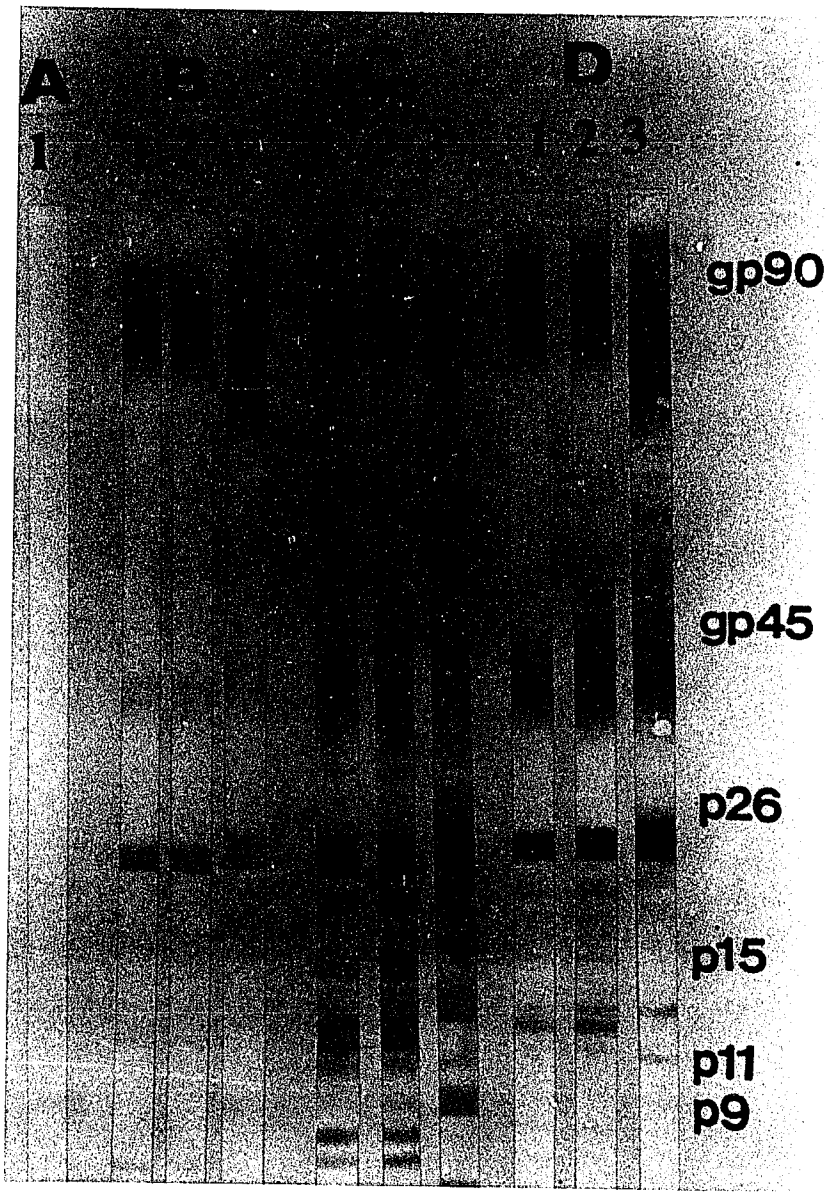

A
B
D
1
1 2 3
gp90
gp45
p26
p15
p11
p9

Fig. 3: Western blot reactivity patterns of sera from naturally infected horses. The protein specificities of four serum samples were tested at 1:50 dilution. Normal horse serum is tested against prototype EIAV in lane 0. Sera from four naturally infected horses are tested in lanes 1-4 against prototype EIAV (panel A) and isolates A/1 (panel B) and A/4 (panel C), respectively.

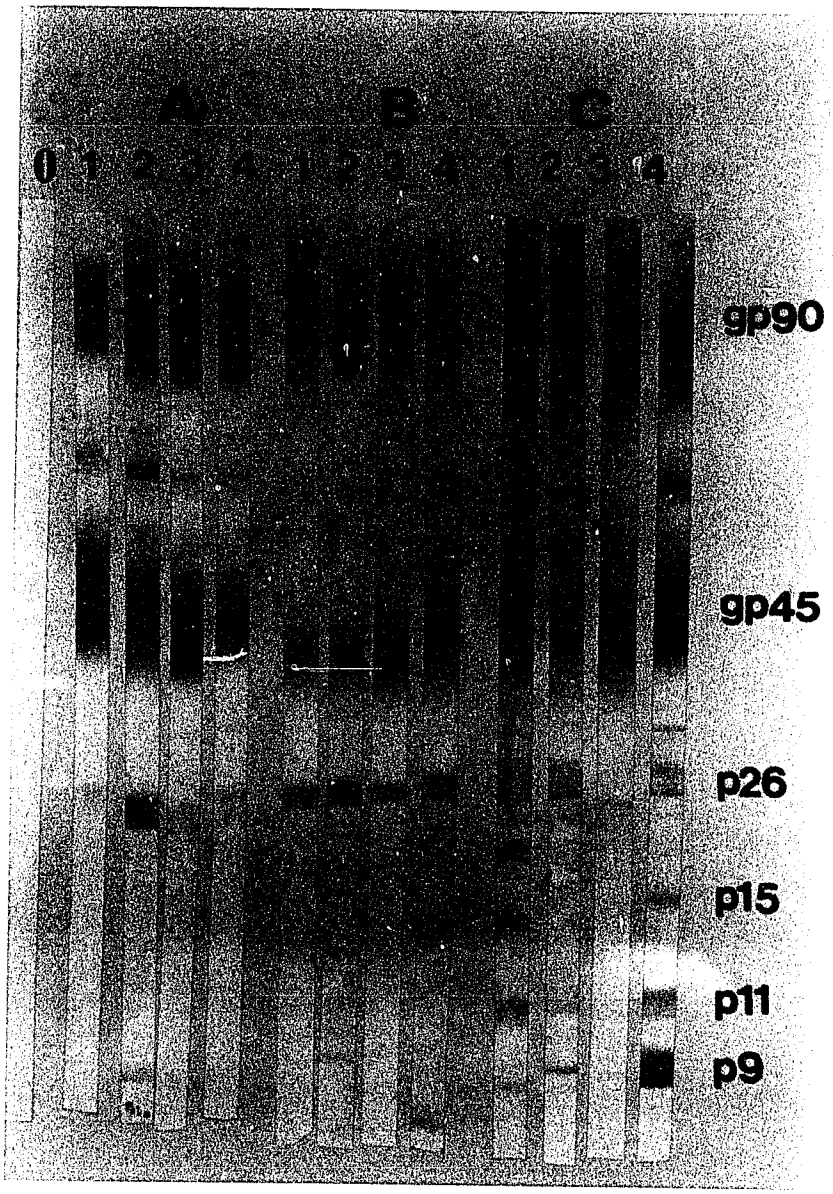

A
B
C
0 1 2 3 4 1 2 3 4 1 2 3 4
gp90
gp45
p26
p15
p11
p9

Fig. 4. Tryptic peptide maps of ^{125}I-gp90 (row A) and ^{125}I-gp45 (row B) isolated from EIAV strains A/1, A/2, A/3 and A/4. Peptide additions (Arrows) and deletions (circles) indicate peptide map differences for each isolate when compared with that of immediate predecessor, i.e. A/2 is compared with A/1, A/3 with A/2 etc.

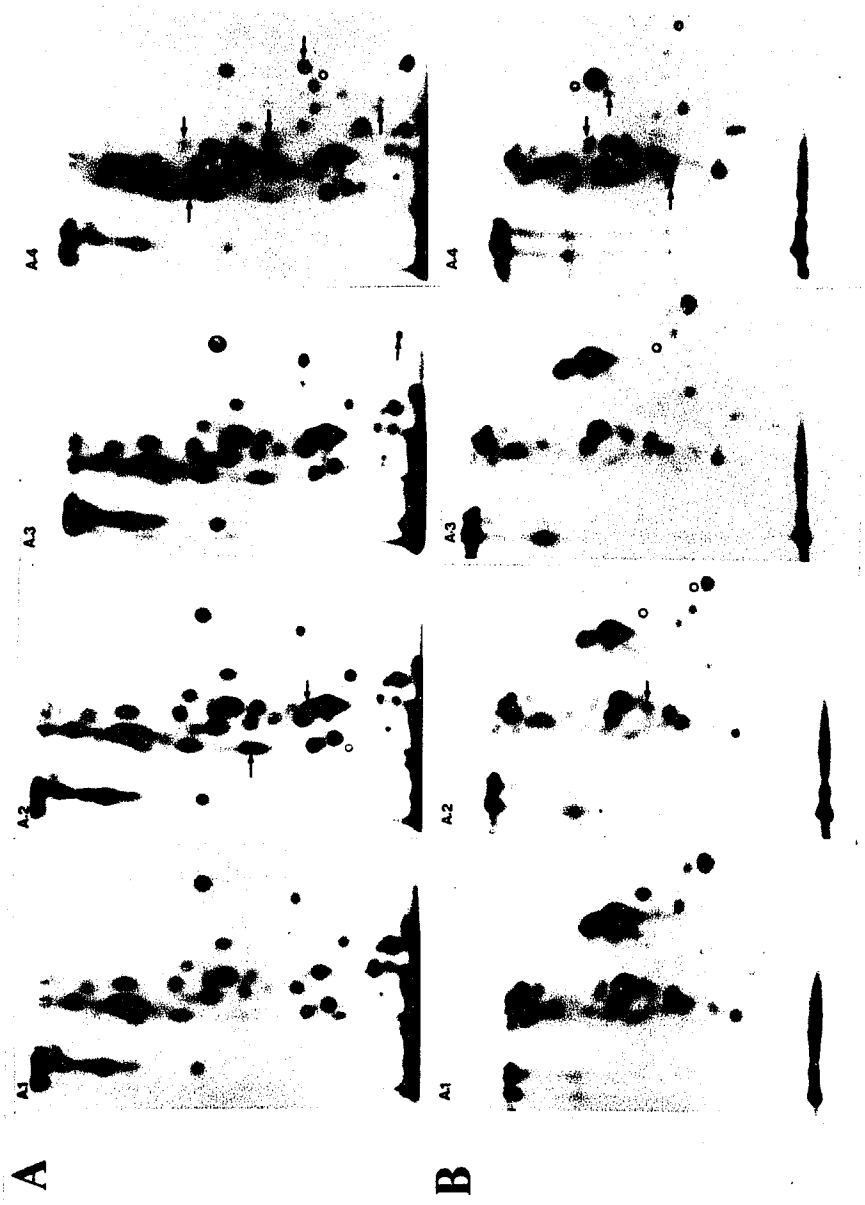

A
B
A1
A2
A3
A4
A1
A2
A3
A4

Table 1: IgG levels to EIAV glycoproteins and major core protein present in early (35 DPI) and late (203 DPI) sera by Western blot.

Viral protein	Percentage in virion*	LEVELS OF IgG	
		Early serum	Late serum
gp90	6.0	3.3[a]	4.2
gp45	3.0	2.0	3.7
p26	45.0	3.3	4.2

* adapted from Parekh et al (25).

[a] Log_{10} of the reciprocal of highest serum dilution positive for specific IgG binding.

Table 2: The protein specificities of MAbs to isolate A/1 and their reactivity with EIAV isolates in Western blots.

	VIRAL	VIRUS ISOLATES							
MAb[a]	PROTEIN	**A/1**	**A/2**	**A/3**	**A/4**	**B/1**	**B/2**	**C/1**	**P[b]**
F1-120	gp90	+	+	+	+	+	+	+	+
F1-123	gp90	+	+	+	+	+	+	+	+
F1-102	gp45	+	+	+	+	+	+	+	+
F1-106	gp45	+	+	+	+	+	+	+	+
F1-110	gp45	+	+	+	+	+	+	+	+

[a] supernatants from hybridoma cell cultures were used at 1:4 dilutions.

[b] prototype cell adapted strain of EIAV.

Table 3: Immunoblot patterns of gp90- and gp45-specific MAbs with polypeptides of seven EIAV isolates including prototype EIAV (P).

	VIRUS ISOLATES							
EPITOPES[a]	**P**	**A/1**	**A/2**	**A/3**	**A/4**	**B/1**	**B/2**	**C/1**
90-A	+	+	+	+	+	+	+	+
90-B	+	-	+	+	-	+	+	+
90-C	+	+	+	+	-	+	-	+
90-D	+	+	+	+	-	+	-	+
90-E	+	-	-	-	-	-	-	-
90-F	+	-	+	+	-	+	-	+
45-A	+	+	+	+	+	+	+	+
45-B	+	-	+	+	+	+	+	+

a - epitopes in gp90 and gp45 (11)

Table 4. Neutralization of EIAV isolates by sera collected from pony A on different days post-inoculation (DPI).

	SERUM COLLECTION DAY				
EIAV-ISOLATE	35*	67	90	203	279
A/1 (16[a])	1.5[b]	3.5	3.5	3.0	3.5
A/2 (46[a])	1.5	3.0	3.0	2.0	2.0
A/3 (75[a])	1.5	1.5	2.5	2.5	2.0
A/4 (107[a])	1.0	1.0	1.5	2.5	2.5

* DPI serum collected

[a] DPI virus isolated

[b] Log_{10} neutralization index (1.7 positive)

Table 5. Neutralization of EIAV isolates by serum collected from pony C that had only one clinical episode of EIA.

		SERUM COLLECTION DAY	
EIAV PASSAGE	VIRUS ISOLATE	35[a]	438
P0*	Prototype	1.5	3.0
P2	P2-1	1.0	3.5
	P2-6	1.0	3.5
P3	A/1	1.5[b]	3.0
	A/2	1.5	2.0
	A/3	1.5	2.0
	A/4	1.0	1.5
	B/1	1.5	2.5
	B/2	1.5	2.0
	C/1	1.0	3.5

* EIAV passage in Shetland ponies

[a] DPI serum collected

[b] Log_{10} neutralization index (1.7 positive)

CHAPTER THREE

IN VITRO ISOLATION OF A NEUTRALIZATION ESCAPE MUTANT OF EQUINE INFECTIOUS ANEMIA VIRUS (EIAV).

SUMMARY

An equine infectious anemia virus isolate (A/1) from the plasma of a pony with clinical signs of equine infectious anemia was serially propagated in fetal equine kidney cell cultures in the presence of neutralizing convalescent serum. After 13 passages, an escape mutant (A/1E) was isolated which resisted neutralization by the homologous serum. Resistance to neutralization of A/1E was associated with loss of epitope(s) in the major envelope glycoprotein, gp90. Our data confirmed that antigenic variation is an important mechanism for the persistence of this virus in the presence of neutralizing serum.

INTRODUCTION

Lentiviruses of man and animals cause persistent infections in their respective hosts. The equine lentivirus, equine infectious anemia virus (EIAV), causes a persistent infection often characterized by recurrent episodes of acute illness marked by high fever (2). Besides persisting in their hosts as integrated proviruses, lentiviruses may persist in immunologically responsive hosts by undergoing antigenic variation. The occurrence of genomic and antigenic variation in EIAV during persistent infections in horses has been documented using a variety of serological and biochemical assays (3, 6, 8, 9, 10, 11). Analysis of virus isolates recovered from sequential febrile episodes with a panel of monoclonal antibodies (MAbs) has documented the presence of common and variable epitopes in the major envelope glycoprotein of EIAV, gp90 (3). Nucleotide sequence data has revealed a high mutation rate of this lentivirus genome during persistent infections (10). It is generally thought that neutralizing antibodies aid in the selection of new antigenic virus variants during persistent infections. In infections with EIAV, serologically distinct variants emerge possibly through immune selection pressure operating on random viral genome mutations. To assess the possible role of neutralizing antibody in the emergence of new virus variants, we generated a neutralization escape mutant of EIAV and demonstrated that resistance to neutralization by immune serum was associated with loss of at least one epitope in the major envelope glycoprotein, gp90.

To generate a neutralization escape mutant, a third passage isolate of EIAV (A/1) was recovered by end-point dilution of plasma from pony A during the first febrile episode in fetal equine kidney (FEK) cells (11). This isolate was serially propagated in FEK cells in the presence of homologous serum. As a plaque assay does not exist for this virus, the end-point dilution method was used to recover the predominant virus population present in the plasma. The virus (A/1) and the homologous serum used in this study were collected from pony A on 16 and 203 days post-inoculation (DPI) respectively (11). The homologous serum had a neutralization index of 3.5 against isolate A/1. The conditions for the propagation of FEK cells have previously been described (7). In this study, the FEK cells (grown in 25 cm^2 tissue culture flasks) were inoculated with A/1 at a multiplicity of infection of 0.01 and incubated at 37^0C for 1 h. After virus adsorption, cell cultures were rinsed with warm phosphate buffered saline (PBS, pH 7.2) and 5 ml of maintenance medium (MEM + 3% fetal calf serum) were added. After 72 h, heat inactivated (56^0C, 1 h) homologous serum was added (final concentration, 10%) and incubation at 37^0C was continued for 3 weeks. Clarified super-natant medium obtained after 3 weeks was used as inoculum for the serial passage of A/1 in FEK cell cultures in the presence of homologous serum. During the subsequent passages, virus-serum mixtures were inoculated onto FEK cell monolayers and maintenance medium containing immune serum was added after virus adsorption without any rinsing. Thus, in a manner analogous to the conditions that exist in persistently infected horses, the homologous serum was maintained in the cultures at all times during the subsequent passages of A/1. The A/1 isolate of EIAV was

propagated in the presence of homologous serum for a total of 13 passages. The ability of the homologous serum to neutralize A/1 at different levels of passage was tested in the alpha method of virus neutralization. During the final passage in the selecting serum, the virus was preincubated with an equal volume of undiluted serum and inoculated onto FEK cells. The recovered virus was "cloned" by end-point dilution in FEK cell cultures and designated as A/1E to denote a neutralization escape mutant derived from isolate A/1. The serological relatedness between A/1 and A/1E was assessed by virus neutralization using homologous and heterologous sera. The heterologous serum was collected on 438 DPI from pony C which had received the same inoculum as pony A (11). The details for virus neutralization are described elsewhere (11). Briefly, serial 10-fold dilutions of each virus were mixed with a standard dilution (1:4 in MEM) of heat inactivated (56^0C, 1 h) serum. The virus-serum mixtures were incubated at 37^0C for 1 h and assayed for residual infectious virus by inoculating duplicate FEK cell monolayers with 1 ml aliquots. Controls consisting of mixtures of virus dilutions with MEM were included for each test. Virus antigens were assayed as previously described (1). The neutralization index for each serum was calculated as the difference in virus titre between samples without serum and that of the virus-serum mixture. A $\log_{10}$ neutralization index (LNI) of 1.7 was considered positive.

The ability of virus isolate A/1 to resist neutralization by the homologous serum was monitored by virus neutralization assay. The LNI of virus A/1 after six serial passages (A/1/6) in the presence of homologous serum was 1.5 lower than that observed for the parent virus

(Table 1). A difference of 1.5 LNI was observed in virus isolates recovered from sequential febrile episodes (11) implying that after six serial passages A/1 in the presence of neutralizing antibodies a significant antigenic alteration had occurred. The virus was further propagated until by 13 passages it completely resisted neutralization by homologous serum and had a modified neutralization pattern by heterologous serum (Table 1). These data indicated that after multiple passages of EIAV in the presence of immune serum the virus had altered antigenic sites which led to inability of homologous serum to neutralize. Partial recognition of the acquired neutralization antigenic sites by heterologous serum suggested either that the specificity of the selecting serum may determine the serological divergence of the new virus strain or that more than one site is involved in virus neutralization. The failure of the selecting serum to neutralize the new virus variant (A/1E) is analogous to what happens in chronic EIA when virus variants emerge in the presence of antibodies with type-specific neutralizing activity and antibodies reacting with group-specific determinants of the envelope glycoproteins (11). New virus populations could emerge during persistent infections in horses as a result of high replicase errors associated with the reverse transcription of virus genome and immune selection pressure. Our data documented for the first time that antibody-mediated immune selection pressure can operate in the emergence of new serological variants of EIAV. The process was gradual and required multiple passages in the selecting serum. During chronic EIA, virus isolates are serologically different even when recovered during sequential disease episodes separated by as little as four weeks (11). The rapid evolution of EIAV during persistent infection in horses

may be due to the lytic infection of highly permissive cells (i.e., monocytes and macrophages) a process that would lead to multiple cycles of virus replication amplifying the replicase errors associated with reverse transcription. Immune selection pressure then operates on a markedly heterogeneous virus population allowing virus variants to emerge rapidly. In contrast, the replication of EIAV in cultured equine fibroblasts is non-lytic and less permissive. During persistent infection in these cells, resistance to homologous superinfection may be mediated by viral receptor down-regulation as a result of the complexing of receptor with viral envelope proteins. This could limit the cycles of virus replication involving reverse transcription. Such a process would limit the rate of viral genome change occurring in persistently infected cells. Antibody selection pressure is then likely to operate on less heterogeneous virus populations and new virus variants would take longer to appear in vitro than in vivo.

The emergence of a serological variant of EIAV after several passages of an end-point diluted virus in the presence of neutralizing serum suggested that the variants recovered during chronic EIA are not all present in the inoculum virus. Our data indicated and confirmed that antigenic variation in the equine lentivirus is one of the mechanisms by which this virus can persist in the presence of immune serum. The alteration of neutralization antigenic sites of EIAV in this variant did not involve the functionally conserved domain which allows replication in equine fibroblasts. This suggested that at least this functionally conserved domain of EIAV is not the target for neutralization of virus infectivity. Moreover, immunoblot analysis of A/1 and A/1E with the

selecting serum (Fig 1) were similar further indicating that antibodies recognizing conserved antigenic domains in gp90 and gp45 are not involved in virus neutralization.

To further characterize the neutralization escape mutant, A/1E was propagated in FEK cell cultures and purified using previously described methods (5). The viral proteins were separated under reducing conditions on 7.5 to 20% gradient gels by SDS-polyacrlyamide gel electrophoresis and transferred to nitrocellulose membranes as described elsewhere (11). All incubations with antibody or with Tris-buffered saline (10mM Tris-HCl, 0.9% NaCl, pH 7.4) (TBS) were at 37^{0}C with agitation. The nitrocellulose strips were blocked for non-specific binding of antibody with 5% non-fat dry milk (4) in TBS for 2 h and incubated for 2 h at 37^{0}C with monoclonal antibodies (MAbs) at 1:20 dilution or equine serum (203 DPI serum) at 1:100 dilution in TBS containing 1% non-fat dry milk. After incubation with MAbs or serum, the strips were washed twice (10 min/wash) in TBS containing 0.05% Tween 20 (SIGMA) and incubated for 1 h with peroxidase labeled goat anti-mouse immunoglobulins (CAPPEL, COOPER BIOMEDICAL, INC., MALVERN, PA) rabbit anti-equine IgG (SIGMA Chemicals, St Louis, MO.) diluted at 1:1000 in TBS containing 1% non-fat dry milk, respectively. The strips were processed for color with 4-chloro-1-naphthol in methanol (Bio-Rad, Richmond, CA).

The production and characterization of MAbs 86, 82, and 115 has previously been described (3). MAb 86 recognizes an epitope (90-A) that exists in all the EIAV isolates tested (3) including A/1 and A/1E, respectively (Fig 2). Our results indicated that during serial propagation of A/1 in homologous serum, the epitopes recognized by MAbs 82 and 115

were apparently altered and could not be detected in A/1E by Western blot. Although MAbs 82 and 115 failed to neutralize A/1, our data demonstrated that resistance to neutralization by equine convalescent serum was associated with loss of epitopes recognized by these MAbs. All three MAbs used in this study reacted with the gp90 of a virus isolate A/2 the in vivo homologue of isolate A/1E) that was recovered from pony A four weeks after the isolation of A/1 (11), indicating that new virus variants emerged from the predominant virus population in a random fashion. The consideration that A/1E is the homologue of A/2 assumes that, in vivo, virus variants evolve from the predominant virus population i.e. A/1. Previous data has shown that gp90 is a target for neutralizing MAbs and during persistent infections rearrangement of epitopes occur in gp90 with minor or no alterations in gp45 (11). Moreover, analyses of sequential and parallel isolates of EIAV recovered from ponies infected with the same virus have shown that the isolates are structurally and genetically different (9, 10, 12). In contrast, antigenic and structural alterations in gp90 and gp45 have not been observed during continued propagation of EIAV in cell cultures in the absence of immune serum (6). The appearance of structurally and antigenically distinct variants of EIAV before the development of neutralizing antibodies, however, indicates that selection of virus variants also involves mechanisms other than by neutralizing antibodies (11).

Our data confirmed previous observations that EIAV is a highly mutable virus. During persistent infection in horses, antibody selection pressure could result in the emergence of novel variants that cause the recurrent febrile episodes associated with chronic EIA in a manner

analogous to our in vitro model. The generation of a virus variant from end-point diluted virus indicates that the viruses that are recovered from persistently infected horses may evolve from a single virus population by random mutations in the viral envelope gene. Selection of this mutant was enhanced by immune selection pressure. Further characterization of this in vitro isolate will be necessary. Preliminary data indicate that the in vitro isolate maintained its virulence and infectivity for ponies as signs of clinical EIA were observed within 10 days of inoculation (Issel, unpublished results). Nucleotide sequence analysis of A/1E and A/1 isolates may help identify sequences that are important in antibody-mediated immune selection pressure either alone or with other immune factors present in infected animals, respectively. Moreover, the generation of virus variants in vitro using either defined sera or monoclonal antibodies and their subsequent genetic analyses will help to identify sequences that frequently change during virus replication. Identification of such sequences may be important in the design of effective immunogens for EIA. The antigenic variation documented for EIAV during persistent infection in horses and in cell culture in the presence of neutralizing antibodies may be a general mechanism for the persistence of lentiviruses of man and animals in immunoresponsive hosts.

REFERENCES

1. AMBORSKI, G. F., JEFFERS, G., AMBORSKI, R. L., ISSEL, C. J.: Equine infectious anemia virus: Development of a simple reproducible method for titrating infectivity of the cell-adapted strain. Am. J. Vet. Res. **40**, 302-304 (1979).
2. CHEEVERS, W. P., McGUIRE, T. C.: Equine Infectious Anemia Virus: Immunopathogenesis and persistence. Rev. Infect. Dis. **7(1)**, 83-88 (1985).
3. HUSSAIN, K. A., ISSEL, C. J., SCHNORR, K. L., RWAMBO, P. M., MONTELARO, R. C.: Antigenic analysis of equine infectious anemia virus (EIAV) variants using monoclonal antibodies: epitopes of glyco-protein 90 (gp90) of EIAV stimulate neutralizing antibodies. J. Virol. **61**, 2956-2961 (1987).
4. JOHNSON, D. A., GAUTSCH, J. W., SPORTSMAN, J. R., ELDER, J. H.: Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Gene Anal. Techn. **3 (1)**, 1-8 (1984).
5. MONTELARO, R. C., LOHREY, N., PAREKH, B., BLAKENEY, E. W., ISSEL, C. J.: Isolation and comparative biochemical properties of the major internal polypeptides of equine infectious anemia virus. J. Virol. **42**, 1029-1038 (1982).
6. MONTELARO, R. C., PAREKH, B., ORREGO, A., & ISSEL, C. J.: Antigenic variation during persistent infection by equine infectious anemia virus, a retrovirus. J. Biol. Chem. **259**, 10539-10544 (1984).
7. ORREGO, A., ISSEL, C. J., MONTELARO, R. C., ADAMS, W. V. Jr.: Virulence and in vitro growth of a cell adapted strain of equine

infectious anemia virus after serial passage in ponies. Am. J. Vet. Res. **43**, 1556-1560 (1982).

8. PAYNE, S. L., PAREKH, B., MONTELARO, R. C., ISSEL, C. J.: Genomic alterations associated with persistent infections by equine infectious anemia virus, a retrovirus. J. Gen. Virol. **65**, 1395-1399 (1984).
9. PAYNE, S. L., SALINOVICH, O., NAUMAN, S. M., ISSEL, C. J., MONTELARO, R. C.: Course and extent of variation of equine infectious anemia virus during parallel persistent infections. J. Virol. **61**, 1266-1270 (1987a).
10. PAYNE, S. L., FANG FU-DE, LIU CHENG-PIN, DHRUVA, B. R., RWAMBO, P., ISSEL, C. J., MONTELARO, R. C.: Antigenic variation and Lentivirus persistence: variations in envelope gene sequences during EIAV infection resemble changes reported for sequential isolates of HIV. Virology **161**, (1987b) in press.
11. RWAMBO, P. M., ISSEL C. J. et al.: Equine infectious anemia virus (EIAV): Humoral responses and antigenic variation during persistent infection in ponies. manuscript in preparation.
12. SALINOVICH, O., PAYNE, S. L., MONTELARO, R. C., HUSSAIN, K. A., ISSEL, C. J., & SCHNORR, K. L.: Rapid emergence of novel antigenic and genomic variants of equine infectious anemia virus during persistent infection. J. Virol. **57**, 71-80 (1986).

Fig 1: Western blot analysis of the neutralization escape mutant A/1E and the parent virus A/1 with the selecting immune serum. Reference EIA negative equine serum is tested in lane 1 against isolate A/1E at 1:50 dilution. Isolates A/1E and A/1 are tested in lanes 2 and 3, respectively with 203 DPI serum at 1: 50 dilution.

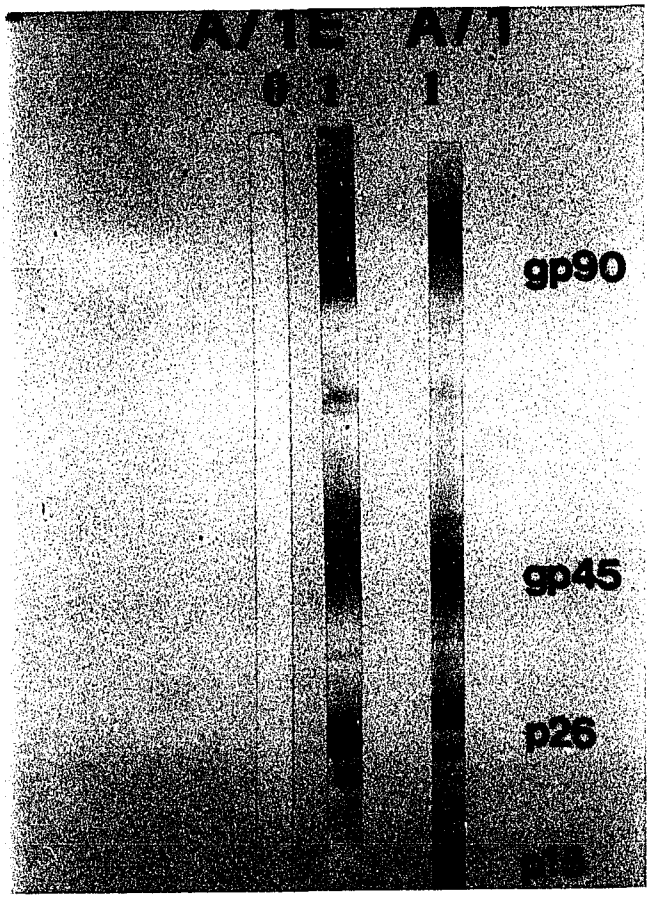


Fig. 2: Western blot analysis of EIAV isolates A/1 (parent) and A/1E (neutralization escape mutant) with a panel of MAbs reactive with the major envelope glycoprotein, gp90. MAbs 86 (lane a), 82 (lane b) and 115 (lane c) were used at 1:10, respectively. Virus A/1 (panel A) and neutralization escape mutant A/1E (panel B).

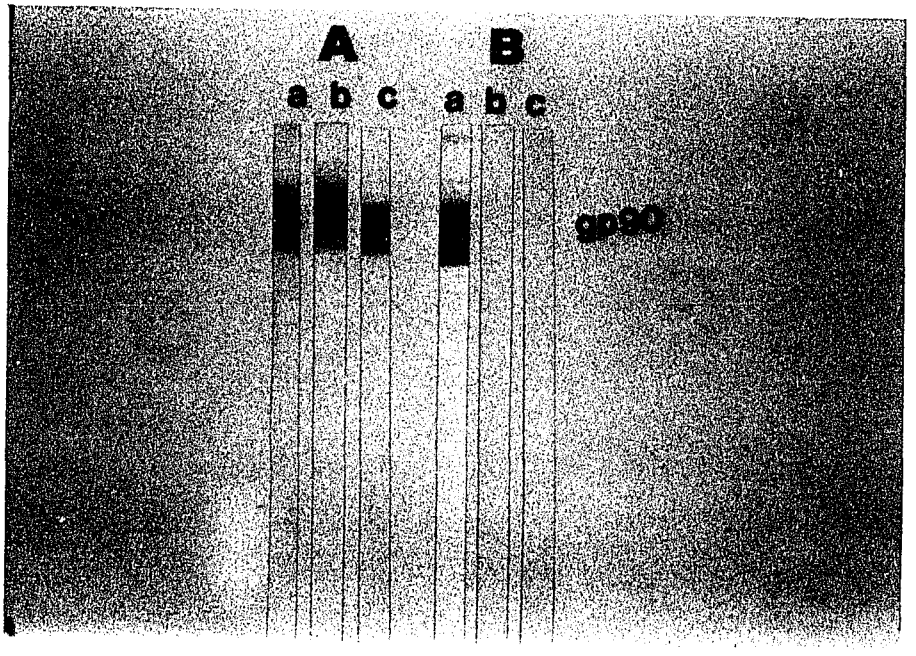


Table 1. Neutralization of EIAV isolates A/1 and A/1E by homologous and heterologous equine convalescent sera.

	VIRUS		
SERUM	A/1	A/1/6*	A/1E+
Homologous	3.5++	2.0	0.7
Heterologous	3.0	ND	2.0

* A/1 virus after 6 passages in the presence of homologous serum.

\+ A/1E isolated after 13 passages of A/1 in the presence of homologous serum.

++ Log_{10} neutralization index (1.7 positive).

ND - not done.

CHAPTER FOUR

APPLICATION OF THE SINGLE-STEP IMMUNOBLOT (SIB) TEST IN DIAGNOSIS OF EQUINE INFECTIOUS ANEMIA AND DETECTION OF ANTIGENS OF EIAV.

SUMMARY

A simple and rapid single-step immunoblot (SIB) assay for antibodies to specific antigens of equine infectious anemia virus (EIAV) was compared with AGID, competition-ELISA and Western blot. A high correlation was observed between these serologic tests in samples with weak positive or positive AGID reactions. Samples with equivocal AGID reactions (8%) tested positive for anti-EIAV antibodies in C-ELISA and Western blots, respectively. These samples could be positively identified with the SIB test using a combination of viral gp90 and p26. In the SIB test, sera were incubated with viral antigens in the presence of "SIB reagent", a protein A-colloidal gold suspension containing a blocker for non-specific binding of antibody. Results to detect equine IgG in Western blots were comparable using peroxidase labeled rabbit anti-equine IgG or SIB reagent. The advantages of SIB include speed, low cost and lack of background problems often seen in Western blots when low serum dilutions are used. The SIB test was found to be applicable for rapid detection of EIAV p26 antigen in fluids from infected cell cultures. The test has potential for wide application in diagnosis of human and animal viral infections.

INTRODUCTION

Equine infectious anemia virus (EIAV) causes a persistent infection in horses that is often characterized by recurrent febrile episodes (3, 5). Horses infected with EIAV produce antibodies to most if not all the viral proteins. Antibodies to EIAV antigens can be detected in the serum of infected horses in a number of serological assays including the agar gel immunodiffusion (AGID) test (4), enzyme linked immunosorbent assay (14) and Western blotting (6). As horses infected with EIAV remain viremic for life and pose a threat to uninfected horses, their rapid and accurate identification is imperative in the control of this disease.

Although AGID test seropositivity correlates very highly with infection, the test lacks the sensitivity to identify all infected seropositive horses (6). In such cases, other testing procedures including competition-ELISA (C-ELISA) and Western immunoblot have proven more sensitive than the AGID test (6). The rapid and accurate diagnosis of virus infections is an important facet in disease control programs. A need exists for continued search of diagnostic procedures that are sensitive, specific, rapid and easy to use. In this study, the use of a rapid, accurate and easy to perform single-step immunoblot (SIB) procedure for the detection of EIAV antibodies was evaluated. The assay is based on the binding of specific serum antibodies to solid-phase adsorbed viral antigens and detection by a protein A gold probe. The test has enormous applicability as antibodies to many antigens/infectious agents can be simultaneously detected.

MATERIALS AND METHODS

Virus antigens: The cell culture adapted Wyoming strain of EIAV (prototype virus) (10) was produced in fetal equine kidney cell cultures and purified using previously described procedures (11). The EIAV major envelope glycoprotein gp90 and the major core protein p26 were purified from density gradient purified virus in a reverse-phase high performance liquid chromatography (HPLC) column as described elsewhere (8). Recombinant p26 covering the Pvu/Dra site in the gag gene was cloned and expressed in E. coli as a fusion protein containing half of p15, entire p26, 8 residues of p11 and 38 amino acids of trp L (Payne, unpublished results). The recombinant antigen was solubilized in 8M urea. The AGID test kits were commercially obtained from Pittman-Moore, Washington Crossing, NJ and C-ELISA test kits were kindly supplied by TechAmerica Corp. Kansas City, MO.

Serum samples: The routine diagnosis of EIA is based on the detection of anti-virus antibodies in AGID or C-ELISA test. Equine sera were collected from a variety of geographical areas in the United States and tested for anti-virus antibody in the AGID test. A total of 115 sera were tested in C-ELISA, Western blots and the SIB test. Five of these sera were from horses previously known to be AGID test positive and which subsequently became equivocal or negative AGID reactors. International reference serum (IRS) for EIA was obtained through Dr. Jim Pearson (USDA Veterinary Services Laboratory, Ames Iowa) and was used in all the four serologic tests for comparison. EIA positive serum (Lady)

with a strong AGID reaction, sera with weak AGID reactions and pooled EIA negative serum were included as controls. Rabbit anti-p26 serum was prepared by immunizing rabbits with HPLC purified EIAV p26.

AGID test and C-ELISA: The AGID test was conducted using the approved protocols (12) and was read after 24 h according to manufucturer's criteria. Samples which caused a deviation of the reference positive serum precipitation line similar to that observed with IRS, but did not form a line in common with reference positive serum, were judged as weak positive reactions. Samples which led to a disagreement in the interpretation of the reaction by three trained individuals were regarded as equivocal. Those samples which formed a line of precipitation in common with the reference line were interpreted as positive. The C-ELISA test was performed according to the manufacturer's procedures (TechAmerica Corp., Kansas City, MO). Samples which visually gave an equal or lower intensity of color than the reference positive sample were considered positive.

Protein A-gold conjugation: Colloidal gold particles of uniform size (15 or 30 nm) were produced by reduction of 0.01% tetrachlorouric acid with 1% sodium citrate according to previously described methods (15). The pH of the colloidal gold sol was adjusted to approximately 7.0 with 0.01M K_2CO_3 and the optimum amount of Staphylococcus aureus protein A (SIGMA Chemicals, St. Louis, MO) in 0.05M Tris buffer (pH 7.4) added. After testing the stability of protein A-gold with 10% NaCl (a blue precipitate forms upon addition of NaCl when the amount of protein

present is insufficient to protect the colloidal gold from salt-induced flocculation), Tris buffer containing 0.1% polyethylene glycol 20M (PEG 20M) was added. The protein A gold solution in 0.05M Tris buffer (pH 7.4) containing 2.5-4% non-fat dry milk (Carnation) as a blocker was used as the detecting reagent. The detecting reagent (subsequently referred to as the SIB reagent) could be stored at 4^0C for up to 1 year.

Determination of optimum amount of antigen and serum dilution for single-step immunoblot (SIB): Density gradient purified virus was treated with 4M KSCN (v/v 1:10) for 15 minutes at 37^0C. The disrupted virus, p26, recombinant p26 and gp90 were diluted in Tris-HCl (0.05M, pH 7.4) to include a concentration range of 200ng/Ìl to 1.5ng/Ìl. The antigen dilutions were spotted in 1Ìl aliquots on a support matrix (nitrocellulose membrane, 0.45um, Schleicher and Schuell, Keene, NH), incubated at 56^0C for 20 minutes to dry and fix antigen, and placed into test-tubes containing the SIB reagent. Each panel of antigen dilutions was tested against sera with negative, strong or weak AGID reactions for virus specific antibodies, respectively, at three final dilutions: 1:10, 1:100 and 1:1000. The appropriate volume of each serum was added directly to the SIB reagent containing the specific antigen(s). The antigen preparations were also incubated with the SIB reagent but without serum as control for non-specific binding of colloidal gold. The tests were incubated at room temperature with agitation. The development of a colored spot at the site of antigen indicated the presence of specific antibody. As rabbit IgG binds with protein A with high affinity and the SIB reactions occur faster, rabbit anti-p26 serum was used for assessing

activity of the SIB reagent.

SIB test for EIAV antibody: For detection of antibodies to EIAV in the sera of test horses, HPLC-p26 (40ng/Ìl) in Tris-HCl buffer (0.05M, pH 7.4) was spotted on the support matrix as described above and incubated in the SIB reagent containing a 1:100 final dilution of test serum. Sera with weak or equivocal AGID or C-ELISA reactions were tested in SIB using a panel of viral p26, recombinant p26 and gp90 blotted on the same strip of nitrocellulose membrane.

Western blot: EIAV proteins were separated on discontinuous (4% stacking gel), 7.5 to 20% gradient gels by SDS-PAGE under reducing conditions (9). The separated viral proteins were electroblotted to nitrocellulose membranes (0.45Ìm, Schleicher and Schuell) according to previously described methods (2). Nitrocellulose membranes containing separated EIAV proteins were incubated with 5% non-fat dry milk (7) in Tris buffered saline (TBS) (10mM Tris, 0.9% NaCl, pH 7.4) for 2 h at 37^{0}C. Serum samples (n=78) were tested against prototype EIAV and two antigenically different strains of EIAV (isolates A/1 and A/4) isolated from a pony during recurrent febrile episodes. All serum samples were diluted in TBS containing 1% non-fat dry milk and incubations and washes were done at 37^{0}C with agitation. The membranes were then incubated 2 h with test sera (1:50), washed twice (10 min/wash) in TBS containing 0.05% Tween 20 (SIGMA) and incubated for 1 h with peroxidase labeled rabbit-anti horse IgG (1:3000; MILES-YEDA LTD, ISRAEL). After two washes (10 min in TBS/0.05% Tween-20 and 10 min in TBS) bound

antibody was detected with 4-chloro-1-naphthol (Bio-Rad, Richmond, CA) in TBS. Specific viral antibodies were also detected in the SIB test in which case the nitrocellulose membranes containing separated EIAV proteins were incubated for 2-4 h in SIB reagent containing 5% (w/v) non-fat dry milk and 1:100 of the test serum.

Detection of EIAV antigen in the SIB test: Supernatant media from infected and uninfected cell cultures were treated with 40% polyethylene glycol (PEG) and assayed for EIAV antigen essentially as previously described (1). For detection of EIAV antigens in the SIB test, supernatant media from known infected and uninfected cell cultures were clarified at 1,000 x g, treated with 40% PEG for 1 h and precipitated by centrifugation at 1,500 x g for 1 h. The precipitated materials were resuspended in 50Ìl of borate buffer (0.05M, pH 8.6), 8M urea, 5% SDS or 4M KSCN. An equal volume of di-ethyl ether was added to the borate buffer mixture. For disruption of virus, the mixtures were incubated with these reagents at 37^0C for 15 minutes. Aliquots of each sample (1Ìl) were then spotted on nitrocellulose membrane and incubated at 56^0C for 20 minutes for protein binding. The membranes containing test antigens and 40ng of p26 (positive antigen control) were incubated in SIB reagent containing 1:100 of rabbit anti-p26 serum.

RESULTS

Optimum conditions for SIB test.

The optimum concentration for each antigen preparation was determined in a checker-board titration of antigen and EIA positive or negative sera. An antigen concentration which gave positive reactions against sera with weak AGID reactions and negative reactions with reference EIA negative serum was selected as optimum for the test. Detergent disrupted EIAV showed low specificity for anti-virus antibody as some sera negative in AGID and Western blot tested positive. The concentrations of HPLC-p26 and recombinant p26 that gave high specificity and strong intensity of color reactions were 40ng and 200ng, respectively (Table 1). For sera that tested strongly in AGID, as little as 3ng of viral p26 and 12ng of recombinant p26, respectively, could be used (Table 1). Purified gp90 was used at 50ng for sera with positive AGID reactions and 100ng for sera with weak positive AGID reactions. The kinetics of the SIB test were different for equine and rabbit sera and were influenced by the serum dilution and the relative amounts of EIAV specific antibodies in each serum (Table 2). The influence of species and serum dilution on the kinetics of the reaction was greatest for the equine serum probably as a result of the low affinity of protein A for equine IgG compared with that of rabbit. From the kinetics data, an optimum serum dilution of 1:100 was selected for equine sera. For rabbit serum, dilutions greater than 1:100 could be used effectively. With these standards, the SIB test using protein A-colloidal gold conjugate was used to detect antibodies to EIAV in equine serum with results evident within

4 h in all but sera with weakest positive AGID reactions.

Detection of anti-EIAV antibodies in AGID, C-ELISA and Western blot.

All serum samples were initially tested by AGID and C-ELISA. The samples were grouped as negative, equivocal, weak positive or positive according to their AGID results (Table 3). All the samples with equivocal AGID reactions gave positive reactions in C-ELISA and had reactivity to EIAV gp90 in Western blots (Fig. lanes 3, 4, 5, 7, 8 and 9). Five of the six samples also had reactivity to EIAV gp45 and p26. It was necessary to dilute serum samples fifty fold to eliminate background staining in Western blot. Two samples (10165 and 10126) from a horse (mare 4) with a history of AGID test positive results were AGID test negative but positive in Western blot at the time of this study (Fig 1, lanes 3 & 9). Sample 10165 was also positive in C-ELISA and had comparable activity to that of IRS in Western blot (Fig. 1, lanes 2 & 3). All samples with equivocal AGID reactions and 2 samples with negative AGID reactions had high activity to gp90 in Western blot.

There was complete agreement in AGID, C-ELISA and Western blot results for groups of samples with weak AGID results (Table 1). All 63 samples had detectable reactions against gp90, gp45 and p26 of prototype EIAV, isolate A/1 and isolate A/4, respectively. Strong activity to gp90 and gp45 and weak activity to p26 was observed at the 1:50 serum dilution used. Differential reactivity to gp45 of the three virus strains was observed with a few of the serum samples with positive AGID results (Fig. 1, lanes 10-12).

The reactivity of equine sera with EIAV proteins separated on SDS-PAGE and blotted to nitrocellulose membranes was also detected in the SIB test. With EIA positive sera, intense color developed within 2-4 h (Fig. 2). Western blots developed using the SIB reagent or peroxidase labeled rabbit anti-equine IgG gave identical results. The reference EIA negative serum had no reactivity and at the 1:100 serum dilution used there was no background staining with the SIB reagent. The colloidal gold gave stable reactions while membranes processed with the peroxidase system faded with storage.

SIB for the diagnosis of EIA.

Analysis of 115 sera in the SIB test showed high correlation between results in the SIB test using HPLC purified p26 (40ng) and the USDA approved diagnostic tests for EIA including the AGID and C-ELISA tests (Table 4). As all the sera with equivocal AGID results would be considered as EIA test-negative by most trained personnel, the six serum samples with equivocal AGID reactions were recorded as negative for comparison (Table 4). There was 100% correlation for all sera with AGID reactions equal to or stronger than that of the International Reference Serum (Table 4). There was also 100% correlation between C-ELISA and the SIB test for six sera with equivocal AGID reactions including AGID test-negative samples from horses with a history of AGID test positive results. Two serum samples negative in AGID and C-ELISA, respectively, tested positive in the SIB test when HPLC purified p26 was used (Table 4). These two samples were also negative in Western blots. The two samples gave no reactions when gp90 and recombinant p26 were used in

the SIB test. EIA positive results were indicated by reactivity with gp90 alone or in combination with p26 (Fig. 3). On the other hand, some sera with equivocal AGID reactions tested positive only with gp90 and recombinant p26 (Fig. 4) and required longer incubation times. The same sera (Flicker and FLA 253) were shown to have antibodies to gp90 and p26 in Western blots (Fig. 1, lanes 4 and 5).

Detection of EIAV antigens.

The presence of EIAV in infected cell cultures was assayed in the SIB test using rabbit serum to purified EIAV p26. Detection of virus antigens in supernatant medium of FEK cell cultures was determined in AGID and the SIB test. The SIB test could yield results within 4 h in contrast to the 48 h usually required for antigen detection by AGID. In persistently infected FEK cell cultures, EIAV p26 was detectable in supernatant fluids without any treatments. However, the detection of p26 early during the infection required pretreatment of clarified supernatant media with PEG and disruption of precipitated virus with chaotropic reagents. Treatment of the precipitate with borate buffer, urea and SDS gave negative or more variable results possibly due to poor antigen binding on the support matrix or inadequate release of the major core protein. Disruption of precipitated virus with 4M KSCN consistently gave positive results when supernatant fluids from infected cultures were tested. Supernatants fluids from uninfected cell cultures did not test positive with normal rabbit serum or rabbit anti-p26 serum. A positive antigen control (40ng of p26) was included in the test to check activity of the SIB reagent and the rate of the reaction, respectively (Table 5).

DISCUSSION

EIA is currently diagnosed by detection of anti-EIAV antibodies in serum of infected horses in AGID and C-ELISA tests (5, 6). These serological tests have been useful in controlling the spread of EIA. In certain cases, however, these tests fail to detect infected horses which appear to have low levels of anti-EIAV antibodies (6). This low sensitivity may result in the erroneous interpretation of the serological results. In these instances, Western blot procedures have proved more sensitive (6).

In this study, several serologic tests were evaluated for detection of anti-EIAV antibodies. There was excellent agreement between AGID, C-ELISA, Western blot and the single-step immunoblot in samples with AGID test reactions equal in intensity to or stronger than the international reference serum. However, some serum samples may have extremely weak AGID reactions and be reported as AGID test-negative during routine testing for EIA. Such samples, designated as having equivocal AGID reactions, had antibodies specific for EIAV gp90, gp45 and p26 as revealed in Western blots. Moreover, all the equivocal AGID reactors were detected in C-ELISA. These results indicated that the AGID is not very sensitive for the detection of low levels of virus-specific antibodies. The Western blot procedure had the highest sensitivity for detection of anti-EIAV antibodies in sera from experimentally or naturally infected horses. Most of the activity was to common antigenic determinants in EIAV gp 90 and gp45; activity to p26 was generally weak and variable.

The use of a rapid single-step immunoblot assay for detection of anti-EIAV antibodies and EIAV antigens was evaluated. In this assay,

specific antibody binds with its Fab-site to antigen attached to nitrocellulose membrane and with its Fc-site to protein A conjugated with colloidal gold. This reaction and the blocking of unsaturated sites on the support matrix occur concurrently. Analysis of 115 sera in this assay using EIAV p26 showed that the test had a comparable level of performance to that of USDA approved tests for EIA. Disrupted purified virus had low specificity probably as a result of cross-reactivity of natural equine antibodies with cellular or serum proteins in the virus preparation. Because of the high sensitivity of the detecting reagent only highly purified antigens should be used to avoid non-specific reactions. Two serum samples negative in AGID, C-ELISA and Western blots gave positive reactions in the SIB test using HPLC purified p26. These "false positive" SIB reactions may be due to either non-specific reactions possibly due to high amounts of pure p26 used or to the recognition of determinants in the purified p26 by antibodies to other equine retroviruses. This is deserving of further study. However, analysis of serum samples with gp90 and recombinant EIAV p26 expressed in E. coli gave the expected results including negative results for the two sera with "false positive" SIB results, indicating that these combination of viral antigens should be used for high sensitivity and specificity of the SIB test. The results also showed that recombinant p26 expressed in E. coli maintained its antigenicity for anti-EIAV antibodies. Moreover, the higher quantities of recombinant p26 required to give intense color change with sera that had weak reactions in AGID test did not lower the specificity of this test. The reactions of two sera with equivocal AGID reactions showed reactivity with gp90 and recombinant p26 indicating that

sensitivity approaching that of the Western blot could be achieved in the SIB test. These results were in agreement with our previous reports indicating that all horses with a history of AGID test-positivity contained antibodies to the virus major envelope glycoprotein which reacted with apparent group-specific determinants (6, 13). Thus, the use of EIAV recombinant p26 and gp90 antigens in the SIB test would provide a sensitive and specific test that is inexpensive, rapid and suitable for any laboratory or field setting.

The Western blot procedure was shown to be highly sensitive for diagnosis of EIA in horses with levels of anti-EIAV antibodies that are below the sensitivity of the AGID and C-ELISA. This procedure, however, is expensive and not practical for the routine diagnosis of EIA and other viral infections. The SIB procedure is not dependent on expensive equipment and can detect presence of specific antibodies to a variety of antigens spotted on the same support matrix. The combination of different viral proteins on the same support matrix, e.g., gp90 and p26, was useful in detecting anti-EIAV antibodies in sera with low levels of antibodies to either protein. Use of combinations of purified preparations that represent the most immunogenic and antigenic proteins of a virus should give SIB test high sensitivity and specificity without the high costs of a conventional Western blot.

Production of EIAV in persistently infected cell cultures was easily monitored in the SIB test using rabbit anti-EIAV p26. The test had high specificity for EIAV antigens and detectable antigens were present in 1µl of supernatant fluids from persistently infected cell cultures with or without pretreatment with PEG and chaotropic reagents. The detection of

EIAV antigens in the AGID test takes at least 48 h. (1). In this study, viral antigens could be detected in the SIB test with anti-p26 serum within 2-5 h. The SIB test therefore presents a rapid method for the detection of anti-EIAV antibodies in serum of horses and EIAV antigens in the supernatant fluids of infected cell cultures, respectively.

In contrast with other immunoassays, the SIB test did not require pre-blocking of unsaturated sites in the antigen support matrix, washes, or second antibody which in most cases needs further processing for either chromogenic reactions or autoradiography. Moreover, the test was easy to interpret as a contrasting color developed at the antigen sites in the presence of specific antibody. The test holds great potential for wide application in the detection of antibodies with specific antigen or detection of antigen with specific antisera, respectively. In viruses where distinct serotypes exist, this test can be of great epidemiological value especially when serotype specific antigens or synthetic peptides are used. Use of colloidal gold conjugated with proteins that have high affinity for the Fc-portion of immunoglobulins from many species would greatly increase the sensitivity, reduce the turnaround time and broaden the spectrum for the use of this assay with sera from different species.

REFERENCES

1. AMBORSKI, G. F., JEFFERS, G., AMBORSKI, R. L., ISSEL, C. J.: Equine infectious anemia virus: Development of a simple reproducible method for titrating infectivity of the cell-adapted strain. Ame. J. Vet. Res. **40**, 302-304 (1979).
2. BURNETTE, W. N.: "Western blotting" electrophoretic transfer of proteins from sodium dodecylsulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112**, 195-203 (1981).
3. CHEEVERS, W. P., McGUIRE, T. C.: Equine Infectious Anemia Virus: Immunopathogenesis and persistence. Rev. Infect. Dis. **7(1)**, 83-88 (1985).
4. COGGINS, L., NORCROSS, N. L.: Immunodiffusion reaction in equine infectious anemia. Cornell Vet. **60**, 330-335 (1970).
5. ISSEL, C.J., COGGINS, L.: Equine infectious anemia: Current knowledge. Ame. J. Vet. Med. Ass. **174**, 727-733 (1979).
6. ISSEL, C. J., RWAMBO, P. M., MONTELARO, R. C.: Evolution of equine infectious anemia diagnostic tests: Recognition of a need for detection of anti-EIAV glycoprotein antibodies. Proc. 5th Int. Conf. Eq. Infect. Dis., University of Kentucky Press, Lexington, KY. (in press).
7. JOHNSON, D. A., GAUTSCH, J. W., SPORTSMAN, J. R., ELDER, J. H.: Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Gene Anal. Techn. **1**, 3-8 (1984).
8. JUDITH, M. B., RAO, V. S. V., ROBEY, W. G., ISSEL, C. J.,

MONTELARO, R. C.: Lentivirus antigen purification and characterization: Isolation of equine infectious anemia virus gag and env proteins in one step by reverse phase HPLC and application to human immunodeficiency virus glycoproteins. manuscript submitted.

9. LAEMMLI, U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**, 680-685 (1970).
10. MALMQUIST, W. A., BARNETTE, D. & BECVAR, C. S.: Production of equine infectious anemia virus antigen in a persistently infected cell line. Arch. Virol. **42**, 361-370 (1973).
11. MONTELARO, R. C., LOHREY, N., PAREKH, B., BLAKENEY, E. W., ISSEL, C.J.: Isolation and comparative biochemical properties of the major internal polypeptides of equine infectious anemia virus. J. Virol. **42**, 1029-1038 (1982).
12. PEARSON, J. E., COGGINS, L.: Protocol for the immunodiffusion (Coggins) test for equine infectious anemia. Proc. Am. Assoc. Vet. Lab. Diag. **18**, 1-13 (1979).
13. RWAMBO, P.M., ISSEL, C.J. HUSSAIN, K.A., ADAMS, W.V. Jr., MONTELARO, R.C.: Equine infectious anemia virus: Humoral responses and antigenic variation during persistent infection. manuscript in preparation.
14. SHANE, B. S., ISSEL, C. J., MONTELARO, R. C.: Enzyme-linked immunosorbent assay for the detection of equine infectious anemia p26 antigen and antibody. J. Gen. Microbiol. **19**, 351-355.
15. SMIT, J., TODD, W. J.: Colloidal gold labels for immunocytochemical analysis of microbes. In Ultrastructure Techniques for Microorganisms, pp. 469-516. Edited by H. C. Aldrich and W. J. Todd, Plenum press,

NY (1986).

Table 1. Sensitivity of SIB test for HPLC purified p26 and recombinant p26 using EIA positive equine sera and EIA negative reference serum.

		AMOUNT OF ANTIGEN (ng/microliter)						
ANTIGEN	AGID[a]	200	100	50	25	12	6	3
p26	S[b]	+	+	+	+	+	+	+
	W	+	+	+	+	-	-	-
	N	-	-	-	-	-	-	-
rec-p26	S	+	+	+	+	+	-	-
	W	+	+	-	-	-	-	-
	N	-	-	-	-	-	-	-

a AGID activity for anti-EIAV antibody indicated as strong (S), weak (W) or negative (N) (see text for detail).

b sera tested at 1:100 dilution in the SIB reagent.

\+ positive for specific antibody after 3 h.

\- negative for specific antibody after 3 h.

Table 2. The kinetics of SIB test for equine and rabbit sera using 40ng of HPLC purified p26.

SERUM SOURCE	DILUTION	TIME IN MINUTES 5	15	60	120	240
HORSE	1:10	-	-	-	1+	2+
(S)	1:100	-	-	1+	1+	3+
	1:1000	-	-	-	1+	1+
HORSE	1:10	-	-	-	-	1+
(W)	1:100	-	-	-	1+	2+
	1:1000	-	-	-	-	-
RABBIT	1:10	1+	2+	3+	4+	4+
(S)	1:100	1+	2+	3+	4+	4+
	1:1000	1+	1+	2+	4+	4+

(S) Reference horse serum or rabbit anti-p26 serum with strong AGID reaction.

(W) Positive horse serum with weak AGID reaction.

(-/+) negative or positive for specific antibody (number of pluses indicate intensity).

Table 3. Correlation of AGID, C-ELISA and Western blot in the detection of anti-EIAV antibodies in equine sera.

		C-ELISA	WESTERN-BLOT[a]		
AGID reactions	Number tested	# Positive	# Positive to: gp90	gp45	p26
Negative	9	1	2	2	2
Equivocal	6	6	6	5	5
Weak positive	38	38	38	38	38
Positive	25	25	25	25	25

[a]: serum samples tested at 1:50 dilution against prototype cell culture adapted EIAV.

Table 4. Comparison of AGID, C-ELISA and SIB serologic assays for detection of anti-EIAV antibodies in equine sera.

AGID[a]	Number	C-ELISA[b]		SIB[c]	
reactions	Tested	# POS.	# NEG.	# POS.	# NEG.
Negative	15	7	8	9	6
Positive	100	100	0	100	0

[a] Interpretation of AGID explained in text.

[b] Sample positive if intensity of color was equal to or lower than that of the positive reference sample.

[c] SIB test using 40ng of HPLC purified p26. Sera were diluted 1:100 in the SIB reagent and the test was scored after 4 h.

Table 5. Detection of EIAV antigens in the supernatant fluids of persistently infected cell cultures.

	ANTIGEN TREATMENT[a]				
Cell Cultures	Borate .05M	Urea 8M	SDS 5%	KSCN 4M	p26[b]
Infected	-	+	++	++	NA
Uninfected	-	-	-	-	NA
NA					+++

[a] Clarified supernatant medium was treated with 40% PEG and processed as described in the text.

[b] HPLC purified p26 (40ng) was included as positive control antigen.

- or + indicate negative or positive results after incubating each preparation with 1:100 dilution of rabbit anti-p26 serum in the SIB reagent. The number of pluses indicate color intensity.

NA- not applicable.

Fig. 1: Western blot profiles of selected serum samples against the prototype cell adapted strain of EIAV are shown in lanes 1-10. The reactivity of one serum sample against prototype EIAV (lane 10), isolate A/1 (lane 11) and isolate A/4 (lane 12) is included for comparison. Serum samples in lanes 1-12 are as follows: (1) reference EIA-negative horse serum; (2) international reference serum; (3) 10165; (4) Flicker; (5) FLA 253; (6) mare 51; (7) mare 40; (8) Stallion 6; (9) 10126; and (10-12) FLA 191.

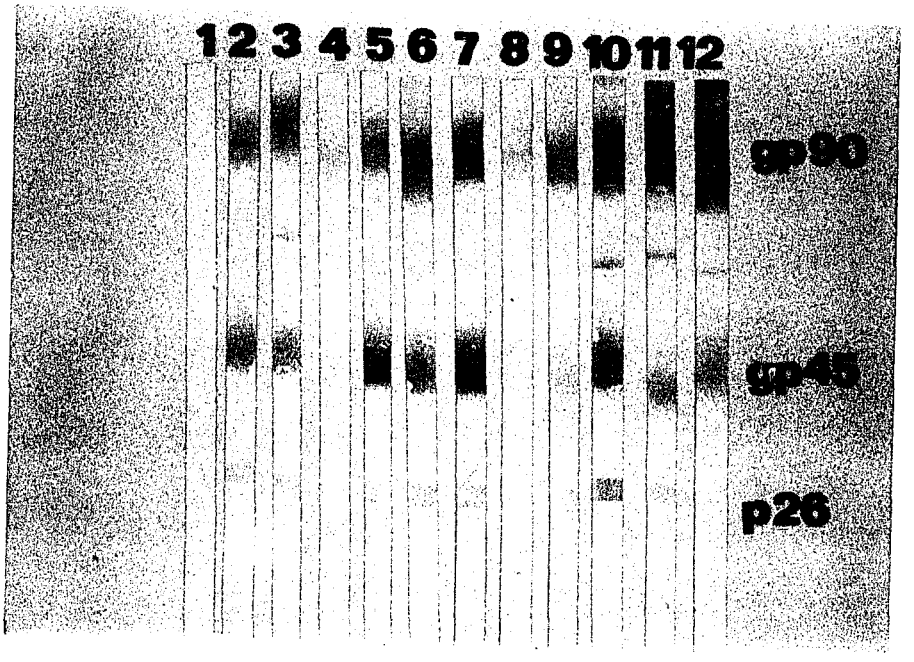


Fig. 2: Western blot analysis of equine sera with the SIB reagent or peroxidase labeled rabbit anti-equine IgG. Virus specific antibodies are tested in panel A with the SIB reagent and panel B with peroxidase labeled rabbit anti-equine IgG, respectively. Reference EIA negative equine serum is tested in lane 0 and EIA positive sera are tested in lanes 1-2 of panels A and B, respectively.

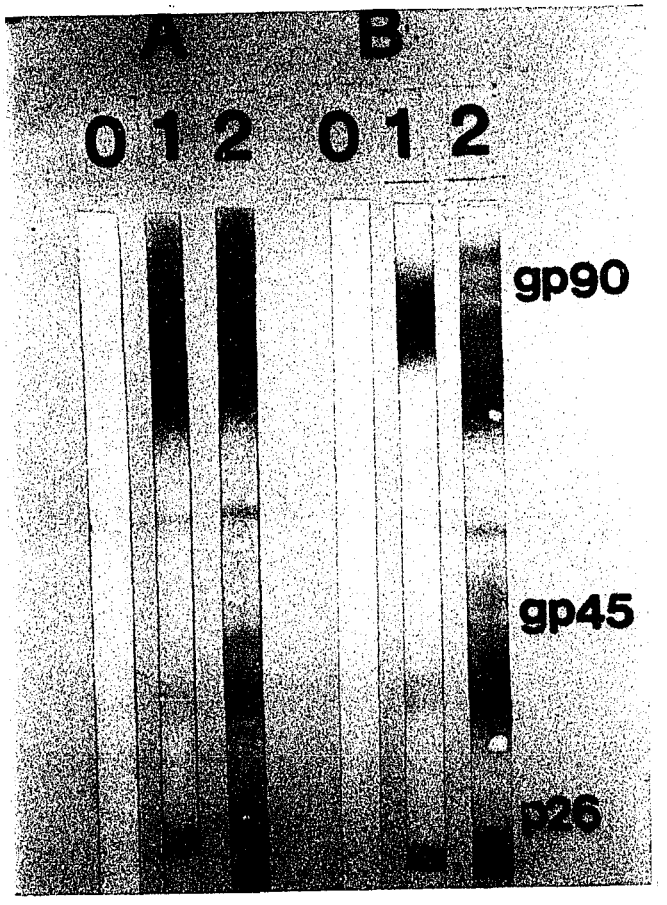


Fig. 3: Detection of antibodies to EIAV gp90 and p26 in SIB test. Sera with weak AGID reactions are tested in lanes 1-4 and sera with strong AGID reactions are tested in lanes 5-8. Reference EIA negative equine serum is tested in lane 0. Each serum is tested against gp90 (top spot), viral p26 (middle spot) and recombinant p26 (rec-p26) (bottom spot). Intense spots at gp90 or p26 sites indicate presence of EIAV specific antibodies.

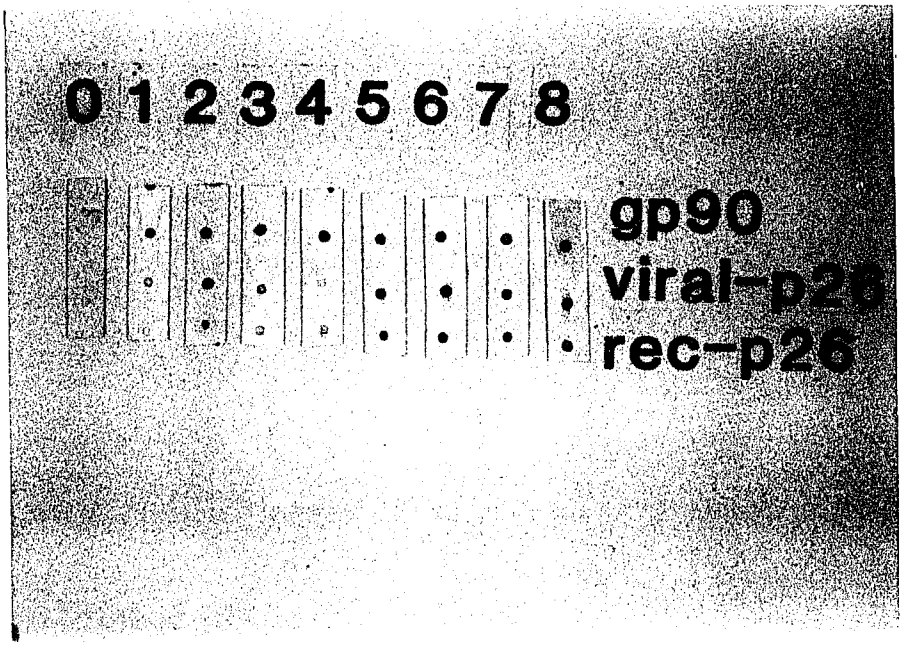
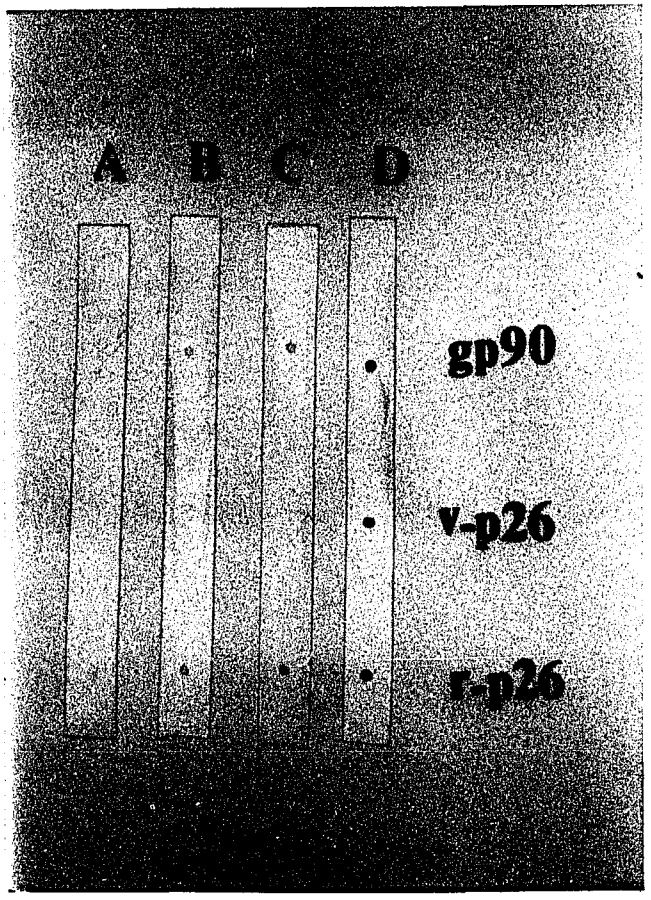


Fig. 4: SIB test analysis of equine sera with equivocal AGID reactions. Reference EIA negative horse serum is tested in lane A and reference positive EIA horse (Lady) serum is tested in lane D, respectively. Sera with equivocal AGID reactions (Flicker and FLA 253) are tested in lanes B and C, respectively. All sera were tested against gp90, HPLC purified p26 (v-p26) and recombinant p26 (r-26).



CHAPTER FIVE

GENERAL DISCUSSION

This study was conducted to characterize variants of EIAV recovered from infected Shetland ponies and variants generated in cell culture, and to determine the nature of specific antibody responses during infection. The course of EIA observed in this study was consistent with previous descriptions (14, 18, 27). Seven virus isolates were recovered from plasma collected during febrile episodes. In published reports isolates A/1-4 were designated as P3.3-1, P3.3-2, P3.3-3 and P3.3-4 for passage level in ponies (P3), animal number (3, 4, 5), and febrile episode (1, 2, etc.) (13, 30).

Recurrent febrile episodes were observed during chronic EIA and viruses recovered from these episodes were shown to be serologically different. The rate of emergence and the number of serologically distinct variants in each animal was different. The demonstration that isolates recovered during febrile episodes 4-5 weeks apart were only neutralized by homologous sera and that definite antigenic changes could be identified with MAbs confirmed that EIAV undergoes rapid antigenic variation. This study extends previous reports which have shown that during persistent infection, EIAV undergoes rapid structural and antigenic alterations in gp90 and gp45 which appear to result from point mutations in the env gene (13, 22, 28, 29, 30, 31). In addition, this study demonstrated that the rapid variation of EIAV observed in persistently infected animals has an immunological basis as the neutralizing specificity of selecting serum appeared to determine the serological divergence of

the in vitro variant.

The generation of an in vitro variant of EIAV suggested that mutant viruses that appear during virus replication require a selecting medium in order to replace parental virus. In the presence of neutralizing antibody, the parental virus and the closely related mutant viruses are neutralized while more divergent mutants continue to replicate. The presence of neutralizing antibodies in infected cell cultures provide a selection pressure that operates on virus variants generated by an independent mechanism of genetic variation of the viral genome. Non-neutralizing antibodies may interfere with virus maturation by causing "patching and capping" of cell membrane-bound viral glycoproteins. However, as the virus can also bud into cytoplasmic vacuoles, the effect of non-neutralizing antibodies may be minimal. As most of the genetic variation is likely to occur during reverse transcription of the viral genome, the replication of virus in cells that are susceptible to lytic-infection will increase the rate at which genome variation occurs. In contrast, the replication of virus in persistently infected cells is likely to limit the rate of genome variation as the majority of the virus probably replicates from proviruses established during the first few rounds of infection when susceptible uninfected cells are still available. Once maximum cell density in culture is achieved and the pool of susceptible cells has been infected, homologous superinfection which is required to maintain cycles of reverse transcription may be prevented by mechanisms which would result in a decrease in expression of viral receptor. Such a process may proceed via receptor interaction with shed viral envelope glycoproteins followed by receptor-ligand internalization, formation of receptor-ligand complexes

between nascent cellular and viral envelope proteins or reduction in the steady state synthesis of the receptor molecules. A similar mechanism of receptor down-regulation occurs in T cells persistently infected with HIV where expression of the HIV receptor (CD4) on cell surface is decreased through reduction of mRNA synthesis and the formation of complexes of available CD4 with viral envelope products (9). The faster rate of emergence of antigenic variants in vivo may thus be explained by the lytic infection of susceptible cells including monocytes and macrophages accompanied by selection pressure operating at both the B- and T-cell epitopes.

The ability of heterologous serum to partially neutralize the in vitro virus variant suggested that either the specificity of the selecting serum determines the serological divergence of the new virus or more than one site is involved in virus neutralization. These possibilities are not mutually exclusive. More than one site is involved in the neutralization of many viruses including influenza virus and HIV and mutants that resist neutralization by one MAb may retain their sensitivity to neutralization by MAbs mapping at different epitopes (10, 35). Results presented in this study demonstrated that antigenic variation allows EIAV to infect host cells in the presence of immune sera. The evolution of new virus variants in vivo may eventually be controlled by the presence of immunological specificities that can accommodate a variety of antigenic alterations. Knowledge of these immunological specificities and the viral antigens that elicit them will be necessary for the design of effective vaccines.

The humoral responses to EIAV during persistent infection were

monitored by AGID, Western blotting and virus neutralization. Specific IgG to gp90 was demonstrable within 2 weeks after infection. The rate of appearance of IgG to gp45 was slower than that observed for gp90 and p26. Although this early IgG had no virus neutralizing activity in vitro, it is likely that in vivo, antibodies binding to these proteins can be protective by complement or cellular dependent mechanisms. Furthermore, specific IgM responses which were not monitored in this study may have important protective roles before the appearance of specific IgG. Type-specific neutralizing antibodies appeared within 2 months after infection. These results are in agreement with previous reports indicating that neutralizing antibodies appeared 45-87 days after infection and peaked 3-5 months after infection (17). The broad neutralizing activity of serum collected late in the infection could have on the one hand resulted from sequential stimulation of the immune system with multiple virus variants as may have been the case with ponies A and B. On the other hand, the specificity of neutralizing antibody may have broadened through repeated stimulation of the immune system with one virus strain as might have been the case with pony C.

Stimulation of the immune system with either sequential virus strains or one virus strain has been shown to elicit broadly neutralizing antibodies in sheep infected with visna virus or in chimpanzees infected with a strain of HIV, respectively (24, 25). However, the facility with which the lentiviruses alter their surface glycoproteins (3, 7, 8, 29, 30) suggests that during persistent infections the immune system is stimulated by "quasispecies" (5) rather than a monotypic strain of virus. The broadening of the humoral response may result from stimulation of the

immune system with a spectrum or "cock tail" of virus variants, which may differ in their replication but not immunogenic potential. The virus populations that predominate in cell culture represent variants capable of replicating extensively under the culture conditions. This may not be the variant that replicates predominantly in vivo where a variety of target cells are likely to be involved. The rate of appearance of specific IgG and recurrences of acute EIA imply that conserved epitopes are not likely targets for protective immune responses. The failure of antibodies binding to these conserved epitopes to control virus replication suggest that: the virus replicates in sites with low concentrations of antibodies; or the virus is predominantly maturing into cytoplasmic vacuoles; or the relevant antigens are not expressed on infected cells. On the other hand, shedding of envelope proteins which has been recognized for other lentiviruses may direct immune mechanism, e.g., cytotoxic T cells, antibody-dependent cell-mediated cytotoxicity (ADCC) and complement away from infected cells. The course of EIA and the early appearance of specific IgG which bind to homologous and heterologous virus isolates but display type-specific neutralizing activity suggests that protective immune responses broaden as a result of stimulation with a spectrum of viruses with modifications at specific B- and probably T- cell epitopes.

The delayed appearance of antibodies capable of neutralizing virus in vitro suggests that other mechanisms may be important in the control of viremia. Leukocytes with direct cytolytic activity appear 14 to 18 days after infection with EIAV (6) suggesting minor role in controlling viremia which in this study peaked 2 to 3 weeks after infection. Equine IgG but not IgG(T) has been shown to mediate antibody dependent cellular

cytotoxicity (ADCC); however, leukocytes from infected horses failed to lyse cells infected with EIAV but lysed heterologous target cells indicating a specific inability for these cells to eliminate EIAV infected cells (6). The absence of effective ADCC during infection with EIAV may explain the inability of horses to control virus replication despite high levels of binding antibodies. On the other hand, it would be of interest to find out whether horses who produce high levels of IgG(T) succumb to recurrent episodes of acute EIA since this subclass of IgG inhibits both ADCC and complement fixation (6, 21). Control of viremia in the absence of neutralizing antibody can be mediated by interferon, fever, and complement with or without specific antibody. In other retrovirus systems, the transmembrane protein has been shown to activate the classical pathway of complement and to bind C1 (1, 4). The acute signs of EIA i.e., fever and depression, are suggestive of increased macrophage function probably in response to opsonized virions. Components of complement and interleukin-1 from activated macrophages may be responsible for fever, depression and the low levels of platelets seen in acute EIA.

The variable course of EIA may be due to many factors including host susceptibility and major histocompatibility genes, i.e. equine leukocyte antigens (ELA), virus strain and intercurrent disease processes. In this study, each pony had a different course of infection after receiving the same plasma. It was difficult to explain the variability in the course of EIA based on the kinetics of humoral response as no difference was detected. Other host factors are likely to be important. It is likely that ponies with appropriate ELA haplotypes become optimally

stimulated by the mosaic of "critical" B- and T-lymphocyte epitopes in the parent virus population. Such horses would remain afebrile after the first episode i.e., pony C. Conversely, ponies with inappropriate ELA haplotypes would be incapable of recognizing all the "critical" epitopes in the parent virus population. Such ponies i.e., ponies A and B, would allow virus mutants to leak through the narrow immune spectrum until a sufficient spectrum of immunological memory is elicited. At present it is difficult to predict the immunological factors that are important in the control of virus replication and cessation of recurrent febrile episodes. It is apparent that humoral responses either appear too late to arrest disease progression or virus is spreading from cell-to-cell causing pathology regardless of the neutralizing specificity of antibodies. The influence of ELA haplotypes on the pathogenesis of EIA can be studied with recipients sharing a single haplotype. This can be achieved by infecting genetically identical horses obtained through embryo splitting.

The ability of lentiviruses to undergo antigenic variation presents a formidable challenge to the development of effective immunogens. The genomes of sequential EIAV isolates demonstrate differences in their nucleotide sequence (29, 30). Within the gp90 region, conserved, variable and hypervariable regions have been identified; most of the amino acid substitutions in the hypervariable region involved potential N-linked glycosylation sites (30). Alterations of potential glycosylation sites may explain the variation in glycopeptide maps previously observed in a set of parallel third passage isolates (31). The variation involving peptide and glycosylation patterns in each virus isolate may alter antigenicity, thus allowing each virus strain to circumvent specific immune factors elicited

by progenitor viruses. In influenza virus, removal of a potential glycosylation site in H5N2 was associated with high virulence as a result of unmasking of a cleavage site (16). It is likely that alteration of glycosylation patterns of EIAV may alter host range, immunogenicity and antigenicity. Of interest in this regard is the apparent resistance of wild-type EIAV to infect cultured equine fibroblasts. The level at which this restriction operates is currently not known. In other lentivirus systems, restriction has been shown to operate at the level of host cell recognition (19, 20). The failure of wild-type virus to replicate in cultured equine fibroblasts suggests that the wild-type virus predominantly replicates in a different cell type in vivo. Different glycosylation patterns in wild-type virus may alter recognition by putative prototype virus receptor on FEK cells. This can be explained in the following hypothetical scenario: during infection of ponies with prototype virus only spontaneous mutants with altered host cell specificity are capable of infecting target cells associated with replication of EIAV. Conversely, only those spontaneous host range mutants are capable of replicating in FEK cells during virus reisolation from plasma. In both directions, only a very minor virus population of spontaneous mutants is initially present. Accordingly, virulence of prototype virus increased with serial passage in ponies (26) probably as a result of predominant virus population gradually shifting from an "FEK-population" to an "in vivo population". A reverse shift in population may occur during virus reisolation in FEK cells which takes at least 6 weeks of culture. The ability of the prototype virus to replicate in this manner suggests a hot-spot genetic defect that is absent in wild-type virus. Clearly a need to explore the in vivo target cells for

EIAV exists and previous data implicating monocyte/macrophages on immunofluorescence data need to be extended with technology capable of distinguishing between passive antigen expression (phagocytosis of viral-antibody complexes) and authentic virus infection i.e. proviral DNA. The isolation of monocytes and lymphocytes from horses with chronic EIA and their fusion with FEK cells can determine whether the restriction of wild-type EIAV to replicate in this cells is at receptor recognition.

In EIA, after a number of clinical episodes, infected horses may die or remain asymptomatic. The eventual cessation of recurrent clinical EIA is thought to have an immunological basis (2). The neutralizing activity of convalescent serum antibody increased with time and was highly cross-reactive late in the infection. This observation leads to the suggestion that given enough immunological stimulation, e.g., immunization with several virus strains, horses would develop broadly reactive immune responses. However, the role of neutralizing antibody in the pathogenesis of lentivirus infections is challenged by the frequent observation that the level of neutralizing antibody does not appear to influence the course of disease. For example, in this study all the ponies died despite the presence of neutralizing antibodies that were detectable as early as 2 months after infection. Similarly, neutralizing antibodies are present in the sera of both asymptomatic HIV seropositive persons and in AIDS patients suggesting that naturally developing neutralizing antibody offer little protection (33, 34). Moreover, immunization of chimpanzees with vaccinia-HIV env recombinants did not avert challenge infection despite the presence of specific antibody and T-cell responses (11). Analysis of the humoral and cellular immune responses in healthy seropositive

individuals (i.e., ponies who have survived for long periods following infection) might shed some light into the nature of long-term protective immune mechanisms. In addition, full knowledge of the interaction of these lentiviruses with other intercurrent pathogens in the disease process may aid in the design of disease management protocols. The recognition that herpes viruses and lentiviruses may possess transactivating genes raises the speculation of potentiation of viral gene expression during dual infection with these viruses and a possible fatal outcome.

The diagnosis of viral infections by detection of specific antibody is limited to the phase of infection when immunological responses are measurable. The time between infection and detection of infection by serology is variable in most viral infections and represents a critical window in disease control measures. Of greatest concern in this regard are the viruses which cause persistent viremia in their hosts since infected individuals can directly or indirectly disseminate virus for long periods prior to diagnosis. For this reason, technology that can directly determine the presence of virus in an individual is most desirable. In the absence of such technology, continued search for simpler, inexpensive and sensitive serological tests is implied. In this study, a single-step immunoblot method was evaluated for its application in the rapid diagnosis of EIA. The SIB test relies on the binding of colloidal gold conjugated with protein A to the Fc- portion of specific antibodies that react with antigens attached to a support matrix. By reacting test sera with both gp90 and p26, a level of sensitivity and specificity similar to that of the approved diagnostic tests for EIA was achieved. This test also proved more efficient in detection of EIAV antigens in the

supernatant fluids of persistently infected cell cultures.

In a limited study, a rabbit was immunized with lentil-lectin purified glycoproteins of prototype EIAV. Immune serum contained titers $10^{3.6}$ in ELISA using purified virus. The antibodies reacted with several virus isolates including prototype EIAV. Immunoblot analysis of this serum and sera from rabbits immunized with whole or detergent disrupted prototype EIAV indicated strong activity to gp90 and low activity to gp45. However, the anti-glycoprotein serum failed to neutralize homologous or heterologous virus isolates. In contrast, sera from rabbits immunized with whole or detergent disrupted virus had high neutralizing activity (Issel, unpublished results). The failure of the rabbit serum to neutralize virus suggested that soluble purified glycoproteins may not possess the epitopes that are important for eliciting neutralizing antibodies. These results indicated that for the desired immune response to be achieved the antigens will have to be presented in a form that ensures native conformations. In a pilot study, treatment of prototype EIAV with disulfide-activating reagents such as 2,2'-dithiobis-m-nitropyridine did not lead to the formation of heterodimers of gp90 and gp45 (data not shown). In other retroviruses, heterodimers of the outer envelope glycoprotein and the transmembrane protein have been shown to elicit more potent protective immune responses than the individual proteins (12, 32). For viruses that cannot form such heterodimers, immunostimulating complexes (ISCOMS) of glycosides with the glycoprotein inserted in a multimeric form (23) would be a better form of antigen presentation.

The demonstration that the hydrophobic amino terminus of gp41 contains a fusogenic domain responsible for the fusion event following

gp120-CD4 binding and that mutations in this region interfered with membrane fusion (15) suggests a more significant role for this protein in the infection process. In other viruses, membrane fusion is a function of the hydrophobic amino terminus of an integral membrane protein that undergoes maturation by proteolytic cleavage, e.g the orthomyxoviruses and paramyxoviruses. It is likely that binding of antibodies to the outer envelope and the transmembrane protein mediate cooperative neutralization which is more potent than that achieved by antibodies to either protein alone. The time of appearance of neutralizing antibodies in horses with EIA suggest that antibodies to gp90 and gp45 may cooperate in virus neutralization. Furthermore, the transmembrane location of gp45 may facilitate antibody dependent complement mediated cytolysis and virolysis. Information on the immunochemistry of the glycoproteins of EIAV and their specific roles in the early events in virus replication cycle will be necessary in designing strategies for production and purification of non-replicative immunogens for control of EIA. As antigenic variation in this virus appears to be unlimited at the population level, a need to determine clusters of immunodominant and more genetically stable variants from the panel of those generated in vivo and in vitro is evident. Trials with inactivated immunodominant virus variants may open the way for the development and use of cock tails of subunit vaccines that will be protective.

REFERENCES

1. BARTHOLOMEW, R. M., ESSER, A. F., MULLER-EBERHARD, H. J.: Lysis of oncornaviruses by human serum: Isolation of the viral complement (C1) receptor and identification as p15E. J. Exp. Med. **147**, 844-853 (1978).
2. CHEEVERS, W. P. & McGUIRE, T. C.: Equine Infectious Anemia Virus: Immunopathogenesis and persistence. Rev. Infect. Dis. **7(1)**, 83-88 (1985).
3. CLEMENTS, J. E., PEDERSEN, F. S., NARAYAN, O., & HASELTINE, W. A.: Genomic changes associated with antigenic variation of visna virus during persistent infection. Proc. Natl. Acad. Sci. U.S.A **77**, 4454-4458 (1980).
4. COOPER, N. R., JENSEN, F. C., WELSH, Jr. R. M., OLDSTONE, M. B. A.: Lysis of RNA tumor viruses by human serum: Direct antibody independent triggering of the classical complement pathway. J. Exp. Med. **144**, 970-984 (1976).
5. EIGEN, M. GARDINER, W., SCHUSTER, P., WINKLER-OSWATITSCH, R.: The origin of genetic information. Sci. Amer. **244**, 88-118 (1980)
6. FUJIMIYA, Y., PERRYMAN, L. E., CRAWFORD, T. B.: Leukocyte cytotoxicity in a persistent virus infection: Presence of direct cytotoxicity but absence of antibody-dependent cellular cytotoxicity in horses infected with equine infectious anemia virus. Inf. Immun. **24**, 628-636 (1979).
7. HAHN, B. H., GONDA. M., SHAW, G. M., POPOVIC, M., HOXIE, J., GALLO, R. C. & WONG-STAAL, F.: Genomic diversity of the AIDS virus HTLV-3: different viruses exhibit greatest diversity in their

envelope genes. Proc. Natl. Acad. Sci. U.S.A. **82**, 4813-4817 (1985).

8. HAHN, B. H., SHAW, G. M., TAYLOR, M. E., REDFIELD, R. R., MARKHAM, P. D., SALAHUDDIN, S. Z., WONG-STAAL, F., GALLO, R. C., PARKS, E. S., & PARKS, W. P.: Genetic variation in HTLV-3/LAV over time in patients with AIDS or at risk for AIDS. Science **232**, 1548-1554 (1986).
9. HOXIE, J. A., ALPERS, J. D., RACKOWSKI, J. L.,HUEBNER, K., HAGGARTY, B. S., CEDARBAUM, A. J., REED, J. C.: Alterations in T4 (CD4) protein and mRNA synthesis in cells infected with HIV. Science **204**, 1123-1127 (1986).
10. HO, D. D., SARNGADHARAN, M. G., HIRSCH, M. S., SCHOOLEY, R. T., ROTA, T. R., KENNENDY, R. C., CHANH, T. C. & SATO, V. L.: Human immunodeficiency virus neutralizing antibodies recognize several conserved domains on the envelope glycoproteins. J. Virol. **61**, 2024-2028 (1987).
11. HU, SHIU-LOK, FULTZ, P. N., McCLURE, H. M., EICHBERG, J. W., THOMAS, E. K., ZARLING, J., SINGHAL, M. C., KOSOWSKI, S. G., TODARO, G.: Effect of immunization with a vaccinia-HIV env recombinant on HIV infection of chimpanzees. Nature **328**, 721-723 (1987).
12. HUNSMANN, G., SCHNEIDER, J., SCHULZ, A.: Immunoprevention of Friend virus-induced erythroleukemia by vaccination with viral envelope glycoprotein complexes. Virology **113**, 602-612 (1981).
13. HUSSAIN, K. A., ISSEL, C. J., SCHNORR, K. L., RWAMBO, P. M. & MONTELARO, R. C.: Antigenic analysis of equine infectious anemia virus (EIAV) variants using monoclonal antibodies: epitopes of glyco-

protein 90 (gp90) of EIAV stimulate neutralizing antibodies. J. Virol. **61**, 2956-2961 (1987).

14. ISSEL, C.J. & COGGINS, L.: Equine infectious anemia: Current knowledge. J. Am. Vet. Med. Assoc. **174**, 727-733 (1979).
15. KAWALSKI, M., POTZ, J., BASIRIPOUR, L., DORFMAN, T., GOH, W. C., TERWILLIGER, E., DAYTON, A., ROSEN, C., HASELTINE, W., SODROSKI, J.: Functional regions of the envelope glycoprotein of human immunodeficiency virus type-1. Science **237**, 1351-1355 (1987).
16. KAWAOKA, Y., NAEVE, C. W., WEBSTER, R. G.: Is virulence of H5N2 influenza virus in chickens associated with loss of carbohydrate from hemagglutinin? Virology **139**, 203 (1984).
17. KONO, Y., KOBAYASHI, K., & FUKUNAGA, Y.: Serological comparison among various strains of equine infectious anemia virus. Arch. Virol. **34**, 202-208 (1971).
18. KONO, Y.: Recurrences of equine infectious anemia. Approaches to understanding of the mechanisms. In Proc. of the 3rd Int. Conf. on Equine Infectious Diseases, pp. 175-186. Basel: S. Karger (1973).
19. LEVY, J. A., SHIMABUKURO, J., MCHUGH, T., CASAVANT, C., STITES, D., & OSHIRO, L.: AIDS-associated retrovirus (ARV) can productively infect other cells besides human T helper cells. Virology **147**, 441-448 (1985).
20. McDOUGAL, J. S., KENNEDY, M. S., SLIGH, J. M., CORT, S. P., MAWLE, A., & NICHOLSON, J. K. A.: Binding of HTLV-3/LAV to $T4^+$ T-cells by a complex of the 110kD viral protein and the T4 molecule. Science **231**, 382-385 (1986).
21. McGUIRE, T. C., HOOSIER, G. L. van, HENSON, T. B.: The

complement-fixation reaction in equine infectious anemia. Demonstration of inhibition by IgG(T). J. Immunol. **107**, 1738-1744 (1971)

22. MONTELARO, R. C., PAREKH, B., ORREGO, A., & ISSEL, C. J.: Antigenic variation during persistent infection by equine infectious anemia virus, a retrovirus. J. Bio. Chem. **259**, 10539-10544 (1984).
23. MOREIN, B. HELENIUS, A., SIMONS, K., PETTERSON, R., KAARIANINEN, L., SCHIRRMACHER, V.: Effective subunit vaccines against an enveloped animal virus. Nature **276**, 715-718 (1978).
24. NARA, P. L., ROBEY, W. G., ARTHUR, L. O., ASHER, D. M., WOLFF, A. V., GIBBS Jr. C. J., GAJDUSEK, D. C., FISCHINGER, P. J.: Persistent infection of chimpanzees with human immunodeficiency virus: Serological responses and properties of reisolated viruses. J. Virol. **61**, 3173-3180 (1987).
25. NARAYAN, O., GRIFFIN, D. E., CLEMENTS, J. E.: Virus mutations during "slow infection": Temporal development and characterization of mutants of visna virus recovered from sheep. J. Gen. Virol. **41**, 343-352 (1978).
26. ORREGO, A., ISSEL, C. J., MONTELARO, R. C. & ADAMS, W. V. Jr.: Virulence and in vitro growth of a cell adapted strain of equine infectious anemia virus after serial passage in ponies. Am. J. Vet. Res. **43**, 1556-1560 (1982).
27. ORREGO, A.: Equine infectious anemia virus: (1) the generation of virus variant candidates by serial passage in Shetland ponies, (2) attempts to titrate the virus by three different methods. Ph. D. dissertation, Louisiana State University (1983).

28. PAYNE, S. L., PAREKH, B., MONTELARO, R. C., & ISSEL, C. J.: Genomic alterations associated with persistent infections by equine infectious anemia virus, a retrovirus. J. Gen. Virol. **65**, 1395-1399 (1984).

29. PAYNE, S. L., SALINOVICH, O., NAUMAN, S. M., ISSEL, C. J., & MONTELARO, R. C.: Course and extent of variation of equine infectious anemia virus during parallel persistent infections. J. Virol. **61**, 1266-1270 (1987a).

30. PAYNE, S. L., FANG FU-DE, LIU CHENG-PIN, DHRUVA, B. R., RWAMBO, P., ISSEL, C. J., & MONTELARO, R. C.: Antigenic variation and Lentivirus persistence: variations in envelope gene sequences during EIAV infection resemble changes reported for sequential isolates of HIV. Virology **161**, " in press" (1987b).

31. SALINOVICH, O., PAYNE, S. L., MONTELARO, R. C., HUSSAIN, K. A., ISSEL, C. J., & SCHNORR, K. L.: Rapid emergence of novel antigenic and genomic variants of equine infectious anemia virus during persistent infection. J. Virol. **57**, 71-80 (1986).

32. SCHNEIDER, J., FALK, H., HUNSMANN, G.: Envelope polypeptides of Friend leukemia virus: purification and structural analysis. J. Virol. **33**, 597-605 (1980).

33. WEBBER, J. N., CLAPHAM, P. R., WEISS, R. A., PARKER, D., ROBERTS, C., DUNCAN, J., WELLER, I., CARNE, C., TEDDER, R. S., PINCHING, A. J., CHEINGSONG-POPOV, R.: Human immunodeficiency virus infection in two cohorts of homosexual men: neutralizing sera and association of anti-gag antibody with prognosis. Lancet **1**, 119-121 (1987).

34. WEISS, R. A., CLAPHMAN, P. R., CHEINGSONG-POPOV, R., DALGLEISH, A. G., CARNE, C. A., WELLER, I. V. D., TEDDLER, R. S.: Neutralization of human T-lymphotropic virus type 3 by sera of AIDS and AIDS-risk patients. Nature **316**, 69-72 (1985).

35. YEWDELL, J. W., CATON, A. J., & GERHARD, W.: Selection of influenza A virus adsorptive mutants by growth in the presence of a mixture of monoclonal anti-hemagglutinin antibodies. J. Virol. **57**, 623-628 (1986).

VITA

Paul Murumba Rwambo, B.V.M.

Date of Birth: July 19, 1954.

Place of Birth: Kandara, Kenya.

Education: B.V.M., University of Nairobi, Kenya, 1980.

Work Experience:

- July, 1980 to December, 1983: Veterinary Research Officer at Wellcome Institute for Research on Foot-and-Mouth Disease, Embakasi, Nairobi, Kenya.

Publications/Presentations:

Antigenic analysis of equine infectious anemia virus (EIAV) by using monoclonal antibodies: Epitopes of glycoprotein gp90 of EIAV stimulate neutralizing antibodies.

Hussain, K. A., Issel, C. J., Schnorr, K. L., Rwambo, P. M., Montelaro, R. C.: J. Virol. **61**, 2956-2961 (1987).

Antigenic variation and lentivirus persistence: Variations in envelope gene sequences during EIAV infection resemble changes reported for sequential isolates of HIV.

Payne, S. L., Fang, Fu-De., Dhruva, B. R., Rwambo, P., Issel, C. J., Montelaro, R. C.: Virology **161**, "in press".

Antigenic mapping of the envelope proteins of equine infectious anemia virus: Identification of a neutralization domain and a conserved region on gp90.

Hussain, K. A., Issel, C. J., Schnorr, K. L., Rwambo, P. M., West, M., Montelaro, R. C.: Arch. Virol. "in press".

Evolution of equine infectious anemia diagnostic tests: Recognition of a need for detection of anti-EIAV glycoprotein antibodies.
Issel, C. J., Rwambo, P. M., Montelaro, R. C.: Proc. 5th Int. Conf. Eq. Infect. Dis., University of Kentucky Press, Lexington, KY. "in press"

Identification of gag precursor of equine infectious anemia virus with monoclonal antibodies to the major viral core protein, p26.
Hussain, K. A., Issel, C. J., Rwambo, P. M., Arnizaut, B. A., Ball, J., Schnorr, K. L., Montelaro, R. C.: manuscript submitted.

Production of cytopathic effect of equine infectious anemia virus in fetal donkey dermal cell cultures.
McManus, J. J., Issel, C. J., Allgood, T. L., Adams, W. V., French, D. D., Montelaro, R. C., Rwambo, P.: 65th Annual Meeting of the Conference of Research Workers in Animal Disease, Chicago, IL. Abstract #43 (1984).

Distinct serotypes of the equine lentivirus isolated from a pony with chronic equine infectious anemia (EIA).
Rwambo, P. M., Issel, C. J., Montelaro, R. C.: Joint meeting of the American Society for Microbiology and Louisiana Biochemistry Society, Shreveport, LA. Abstract #B 3-1 (1986).

Serological characterization of antigenic variants isolated from ponies persistently infected with the equine lentivirus.
Rwambo, P. M., Issel, C. J., Montelaro, R. C., Amborski, G, F.: Animal Disease Research Workers (Southern States), Gainsville, FL. (1985).

Characterization of genomic and antigenic variations during parallel persistent infections with equine infectious anemia virus.
Montelaro, R. C, Salinovich, O., Payne, S., Issel, C., Rwambo, P.: Annual meeting of American Society for Virology, Santa Barbara, CA. (1986).

Early non-neutralizing sera contain antibodies specific for equine infectious anemia virus surface glycoproteins.
Rwambo, P. M, Issel, C. J., Montelaro, R. C., Schnorr, K. L.: Annual meeting of American Society for Virology, Chapel Hill, NC. (1987).

A novel single step immunoblot test for detection of specific antibody.
Issel, C., Todd, B., Rwambo, P.: First equine immunogenetics and Immunology Conference, Tezcuco Plantation, LA. (1987).

A colloidal gold method for veterinary serology.
Rwambo, P. M., Morris, N. R. G., Issel, C. J., Todd, W. J., Barstad, P. A., England, J. J.: First equine immunogenetics and Immunology Conference, Tezcuco Plantation, LA. (1987).

In vitro isolation of a variant of equine infectious anemia virus (EIAV) using neutralizing serum.

Rwambo, P. M., Issel, C. J., Hussain, K. A., Montelaro, R. C.: 68th Annual Meeting of the Conference of Research Workers in Animal Disease, Chicago, IL. Abstract #346 (1987).

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Paul M. Rwambo

Major Field: Veterinary Medical Sciences

Title of Dissertation: Characterization of Equine Infectious Anemia Virus Variants Generated in vivo and in vitro and a Rapid Assay for Virus-Specific Antibody.

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

RC Montelaro

KL Schnorr

GJ Amoroski

M. Gibbert Gelas

WJ Todd

Date of Examination: 11/20/87