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Viral DNA in Horses Infected with Equine Infectious Anemia Virus

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The amount and distribution of viral DNA were established in a horse acutely infected with the Wyoming strain of equine infectious anemia virus (EIAV). The highest concentrations of viral DNA were found in the liver, lymph nodes, bone marrow, and spleen. The kidney, choroid plexus, and peripheral blood leukocytes also contained viral DNA, but at a lower level. It is estimated that at day 16 postinoculation, almost all of the viral DNA was located in the tissues, with the liver alone containing about 90 times more EIAV DNA than the peripheral blood leukocytes did. Assuming a monocyte-macrophage target, each infected cell contained multiple copies of viral DNA (between 6 and 60 copies in liver Kupffer cells). At day 16 postinoculation, most of the EIAV DNA was not integrated into host DNA, but existed in both linear and circular unintegrated forms. In contrast to acute infection, viral DNA was not detectable in tissues from asymptomatic horses with circulating antibody to EIAV.

Equine infectious anemia virus (EIAV) is a lentivirus that is distantly related to the human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (4, 9, 13, 24, 25). Like HIV, EIAV establishes a persistent infection in the host (see references 3 and 22 for reviews), with blood cells remaining infectious after disease-free intervals of many years (6, 11). The principal target and reservoir of EIAV is a leukocyte, probably the macrophage (14, 20, 21). The disease course caused by EIAV is quite variable. Depending on the virus strain and the host, an infected animal may (i) die following a brief acute illness; (ii) recover from an initial clinical episode and have a variable number of additional recrudescences over time, followed by death or recovery; or (iii) remain clinically normal, with the only overt sign of infection being circulating antiviral antibodies. Acute infections involve fever, viremia, weight loss, and leukopenia, and animals become severely anemic after multiple episodes.

Ideally, to determine the molecular basis of virulence, one would like to compare the structures of viral molecular clones that differ in the severity of the disease they cause. To date, however, there are no published reports of infectious molecular clones of EIAV, much less pathogenic ones. The objective is further complicated by the observation that most virus stocks that are pathogenic for the horse cannot be propagated readily in established tissue culture cell lines, although they will replicate in primary horse leukocyte cultures (14, 17). However, recent studies with HIV (8) have suggested that it is wise to minimize or eliminate passage in culture of a virulent virus stock, since selective pressures in culture can be very different from those in the infected host. It therefore appears advisable to derive molecular clones of EIAV directly from infected equine tissue. Before this can be accomplished, it is necessary to assess the quantity and location of viral DNA in an acutely infected horse. That assessment is the subject of this report.

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MATERIALS AND METHODS

Reagents. Equine dermal and kidney cell lines that produce EIAV were established after extended passaging of the Wyoming strain in horse leukocyte cultures (18, 19). Virus from fetal kidney cells were subsequently used to productively infect the canine thymus Cf2th cell line (1). Virus from these cell lines is not pathogenic for the horse.

A replication-defective molecular clone of EIAV from equine dermal cells has been completely sequenced (24, 25). Its only known defects are a premature termination codon in the *pol* gene and a single-base insertion in *env*.

Preparation of leukocytes (buffy coat) from peripheral blood. To prepare leukocytes from an EIAV-infected horse, we collected 50-ml samples of heparinized blood daily through day 16, when the animal was killed. Blood samples were allowed to stand at 4°C for 30 min, whereupon the leukocyte-enriched portion above the erythrocyte layer was removed. This plasma was centrifuged at about 500 × *g* for 15 min, yielding the buffy coat pellet and the supernatant cell-free plasma. DNA was isolated from the cell pellets, and the plasma was analyzed by immunoblotting (see below).

To determine the virus titer in the serum of the infected horse at day 8 postinoculation (p.i.), we prepared leukocytes as above and seeded them in T75 flasks containing 15 ml of medium (RPMI 1640 containing 20% fetal bovine serum, 30% calf serum, and 1% sodium pyruvate) at 2×10^7 cells per ml. Nonadherent cells were removed after 2 h, and virus was applied in serial dilutions as described by Kobayashi and Kono (15). The occurrence of a cytopathic effect within about 12 days was the indicator of virus in the inoculum. Supernatant fluid from cultures showing no cytopathic effect was applied to a fresh culture. If no cytopathic effect was observed in the second culture, fluid was passed to a third culture. Only if this third culture showed no cytopathic effect was the original inoculum scored as negative.

Immunoblotting. For the immunoblot shown in Fig. 1, 2 ml of each plasma sample (see above) was diluted with phosphate-buffered saline, centrifuged at low speed to remove any remaining cells and debris, and then centrifuged at

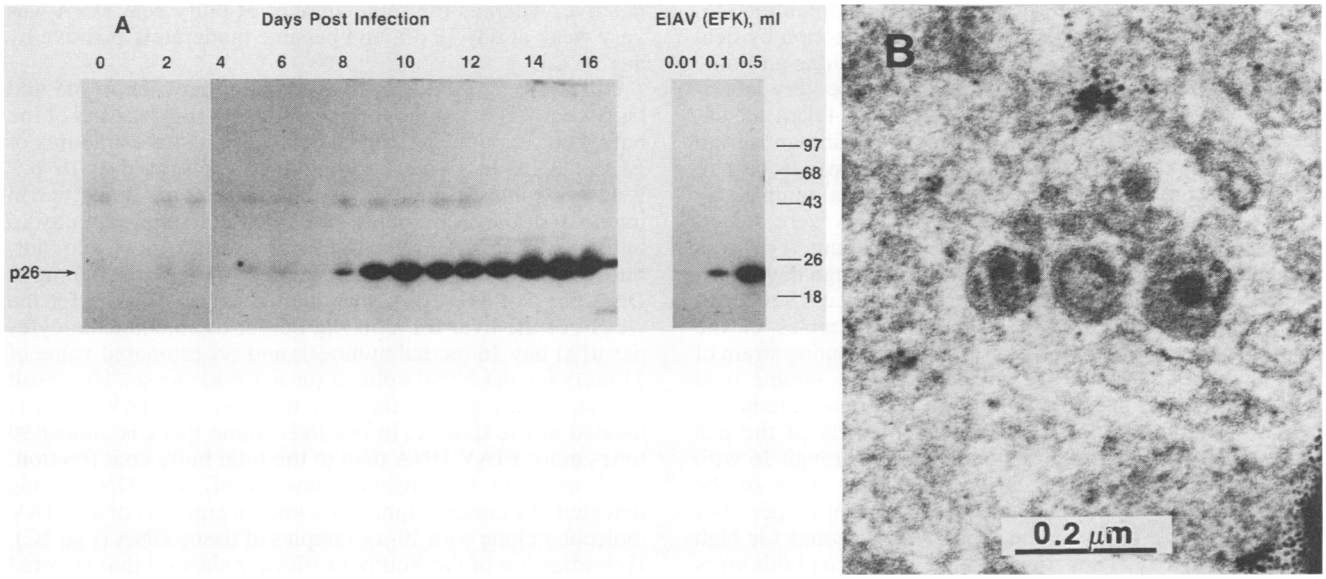

FIG. 1. Appearance of virus following infection with EIAV. (A) Immunoblot of plasma samples. The horse was inoculated with EIAV following collection of the day 0 blood sample. Each lane of the sodium dodecyl sulfate–10 to 20% polyacrylamide gel contained the 100,000 × *g* pellet from 0.5 ml of plasma. The three additional lanes contained sucrose gradient-purified EIAV produced by equine fetal kidney cell cultures (72-h harvest). Dilutions corresponding to the amount of virus derived from 0.01, 0.1, and 0.5 ml of tissue culture fluid were loaded on the gel. The gel was electroblotted onto nitrocellulose, and the filter was incubated with antibody to EIAV p26 (10). Molecular masses (in kilodaltons) of protein size standards are shown on the far right. (B) Detection by electron microscopy of EIAV particles in spleen at 16 days p.i.

100,000 × *g* for 90 min to pellet virus. Each pellet was taken up in gel loading dye, and one-fourth of it (equivalent to all the virus in 0.5 ml of plasma) was loaded onto a sodium dodecyl sulfate–10 to 20% polyacrylamide gel. After electrophoresis, the gel was electroblotted onto nitrocellulose, and the filter was subsequently incubated with a 1:1,000 dilution of antiserum to purified EIAV p26 (10) and then with ^{125}I-protein A at 3×10^5 cpm/ml. The filter was exposed to X-ray film at −70°C for 18 h with an intensifying screen.

Preparation and analysis of DNA. DNA was prepared by lysis of homogenized tissue or of buffy coat fractions in buffer (0.05 M Tris [pH 7.5], 0.2 M sodium chloride, 0.1 M EDTA) containing 0.1% sodium dodecyl sulfate and proteinase K at 100 μg/ml. After incubation at 37°C for several hours, preparations were extracted with buffered phenol and with a 24:1 mixture of chloroform and isoamyl alcohol and banded in a cesium chloride (CsCl) density gradient in the presence of ethidium bromide. The broad DNA band was collected with a pipette, extracted with isopropanol (saturated with CsCl), and dialyzed. When it was examined on a 1% agarose gel, there was no apparent difference among the DNAs in their percentage of high-molecular-weight DNA. Additional DNA from lymph nodes, spleen, and bone marrow was isolated essentially as above, but with ethanol precipitation after phenol-chloroform extraction and without CsCl banding. No differences in hybridization behavior were observed between the two sets of DNA preparations. After digestion with a restriction endonuclease, purified DNAs were electrophoresed in a 1% agarose gel, transferred to nitrocellulose, and probed with a nick-translated molecular clone of EIAV (25). Hybridization was carried out at 42°C in a solution containing 40% formamide, 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and nick-translated ^{32}P-labeled probe at 4×10^6 cpm/ml. The specific activity of the probe was about 10^8 cpm/μg. The most stringent wash was with 0.5× SSC at 65°C. Relative band intensities on the film were determined by densitometric scanning.

Electron microscopy. Tissue which had been quick-frozen and stored at −70°C was added to ice-cold 1.25% buffered glutaraldehyde and allowed to stand overnight. Tissues were embedded, sectioned, and examined by previously described procedures (9).

RESULTS AND DISCUSSION

As virus stock we used serum from a horse infected with the virulent Wyoming strain of EIAV. This stock, provided by C. Issel, Louisiana State University, is known to cause acute disease and early death. This serum (1 ml; 10^6 infectious units/ml in horse leukocyte culture) was inoculated intravenously into a 3-year-old Arabian quarter horse mare. Blood samples were drawn daily, and tissue samples were collected and quick-frozen at day 16 p.i. after the animal had become moribund and was killed. The horse developed a fever at 7 days p.i. and remained febrile (39.4 to 41.1°C) until death. Necropsy demonstrated petechiation and ecchymoses of mucous membranes and serosa, and enlarged hemorrhagic visceral lymph nodes, which are typical of acute EIAV infection. Analysis of blood samples drawn during the course of the infection showed declines in the number of circulating lymphocytes and monocytes and a sharp drop (about sixfold) in the number of circulating platelets; these findings are also typical of EIAV infection (6, 20). As expected for acute infection with virulent EIAV, the horse never became anemic. Antibody to EIAV p26 was first detectable by the agar gel immunodiffusion assay (6) on day 16 p.i.

Appearance of virus in blood and tissue. Development of viremia during EIAV infection has typically been monitored

by tissue culture assay at various times after infection (15, 16, 23). Since the only cells susceptible to infection by field strains are leukocytes and since multiple dilutions and time points are often desired, this procedure can be very laborious and time-consuming. We have therefore taken advantage of the availability of high-titer monospecific antiserum raised against the purified EIAV *gag* protein p26 (10) to monitor the amount of virus in daily plasma samples by immunoblotting (Fig. 1A). Low levels of virus were detectable as early as day 6 p.i., and the maximum level was reached by day 9 p.i. and was maintained through day 16 p.i. To estimate how much virus was present at days 9 through 16 p.i., we included (on the same blot) samples of the avirulent Malmquist derivative (19) of the Wyoming strain of EIAV, which is adapted to and produced by equine fetal kidney cells in culture. We found that for equal volumes of plasma or tissue culture medium, the intensity of the p26 band was about the same (compare days 9 through 16 with EIAV at 0.5 ml). That is, during the acute stage of the disease, horse plasma contains as much virus per unit volume as tissue culture fluid from cells selected for high EIAV productivity. Thus, the extreme virulence of this virus (16 days from initial infection to death) is correlated with massive viral replication, which is unusual for lentivirus infections.

We also measured the infectivity of various dilutions of day 8 p.i. serum samples on horse leukocyte cultures (15) and established a titer of $10^{4.8}$ horse leukocyte infectious doses per ml for day 8 p.i. serum (data not shown). If the approximately 10-fold increase in p26 from day 8 to day 9 seen in the immunoblot is indicative of a 10-fold increase in titer over the same period, the day 9 titer would be about 10^6 horse leukocyte infectious doses per ml. This value would agree well with previous estimates of EIAV titer in serum at the peak of an acute episode (15, 16, 23).

Virus was also detectable in tissue. Frozen samples of spleen (Fig. 1B) and splenic lymph node (day 16 p.i.) were fixed and examined by electron microscopy. Although ultrastructural preservation was poor owing to the freezing and thawing, virus was nevertheless seen in both tissues. Other tissues were not examined.

Levels of viral DNA in infected tissue. Having established an acute EIAV infection exhibiting virus in both blood and tissue, we tested for the presence of EIAV viral DNA. DNA was prepared from various tissues collected when the animal was killed on day 16 p.i. and from leukocyte preparations (buffy coats) obtained from daily blood samples. Restriction enzyme digests of equal amounts of these DNAs were subjected to agarose gel electrophoresis, Southern transfer onto nitrocellulose, and probing with a ^{32}P-labeled molecular clone of EIAV (25).

Hybridizing bands were consistently strongest in liver DNA (Fig. 2A), but DNAs from the spleen, splenic lymph nodes, renal lymph nodes, gastrohepatic lymph nodes, and bone marrow (Fig. 3) were also strongly positive. DNAs from the kidneys, choroid plexus, and buffy coat (days 14, 15, and 16 p.i.) contained less viral DNA, and brain DNA was negative. Thus, at 16 days p.i., the highest concentration of EIAV viral DNA is found in the liver, spleen, lymph nodes, and bone marrow.

To test whether leukocyte DNA contained more EIAV sequences at an earlier time, we examined Southern transfers of the daily samples (Fig. 2B). Hybridizing DNA was first detected in the day 10 p.i. sample, but this and subsequent samples contained little EIAV DNA until days 15 and 16 p.i. Thus, at day 16 p.i., tissue DNA was strongly positive, whereas the same amount of buffy coat DNA was very weak at day 10 p.i. and became moderately positive by day 16 p.i.

Given the difference in hybridization between tissue and buffy coat DNA and the total size of, say, the liver and of the buffy coat fraction, we can calculate the relative amounts of EIAV DNA in tissue and in blood cells at day 16 p.i. Relevant values include (i) a 10× densitometric difference in intensity of the 5.2-kilobase (kb) band in liver and in day 16 buffy coat (Fig. 2A) (this varies somewhat from blot to blot, but 10× is a reasonable middle value); (ii) a total of 2.3 mg of DNA per g of liver; (iii) an estimated value of 7.4 kg for the weight of the liver for a 500-kg horse; (iv) 5,500 leukocytes per μl at day 16 (actual number); and (v) estimated value of 53 liters for the blood volume for a 500-kg horse. The result is that on day 16 p.i. the vast majority of EIAV DNA is located in the tissues. In the liver alone there is almost 90 times more EIAV DNA than in the total buffy coat fraction.

To estimate the absolute amount of viral DNA being detected, we coelectrophoresed known amounts of an EIAV molecular clone with 10-μg samples of tissue DNA (Fig. 2C). Hybridization of the Southern transfer showed that infected liver contained between 0.6 and 6 copies per cell of EIAV DNA (Fig. 2C, compare lane 1 with lanes 14 and 15). However, it is highly unlikely that all cells in the liver are infected. Examination by immunofluorescence of livers from EIAV-infected horses revealed that infected cells are located predominantly within sinusoids and appear to be Kupffer cells (21). Thus, if Kupffer cells constitute 10% of the total cells in the liver (12) and if all of them were infected, each contained on the average between 6 and 60 copies of EIAV viral DNA. If less than 100% of the cells were infected or if significant numbers of them had been destroyed by day 16 p.i., the per-cell levels of DNA in the surviving cells would of course be higher.

EIAV DNA is mostly unintegrated. To determine whether the observed viral DNA is integrated into cellular DNA or is present as unintegrated molecules, we hybridized Southern transfers of tissue DNA which had been digested with restriction enzymes that produce diagnostic fragments. As a reference we used the known restriction map of a Malmquist-Wyoming EIAV molecular clone derived from equine dermal cells (25). Given the heterogeneity found among EIAV isolates (see reference 5 for a review), we expected differences between the reference and the experimental viral DNA for some enzymes, but we assumed that there would be others that would produce comigrating and hence interpretable fragments. This turned out to be the case.

Digestion with an enzyme that cuts the genome only once is a convenient way to distinguish integrated from unintegrated viral DNA, since only unintegrated genomes will yield detectable fragments of predictable sizes. Digestion of tissue DNA with each of three enzymes that cut the reference genome only once (*Sst*I, *Nde*I, and *Pst*I) yielded fragments that must have originated from linear unintegrated DNA (Fig. 3). For example, *Sst*I cuts the reference genome at base 3018 and therefore would produce fragments of 3.0 and 5.2 kb from a linear unintegrated provirus. Fragments of this size were observed in *Sst*I digests of all tissues examined, including the spleen, renal and splenic lymph nodes, kidneys, and bone marrow. *Nde*I cuts the reference genome at base 5725 and therefore would produce fragments of 5.7 and 2.5 kb from a linear unintegrated viral genome. These fragments were observed in each of the three tissue DNAs examined: spleen, renal lymph node, and bone marrow.

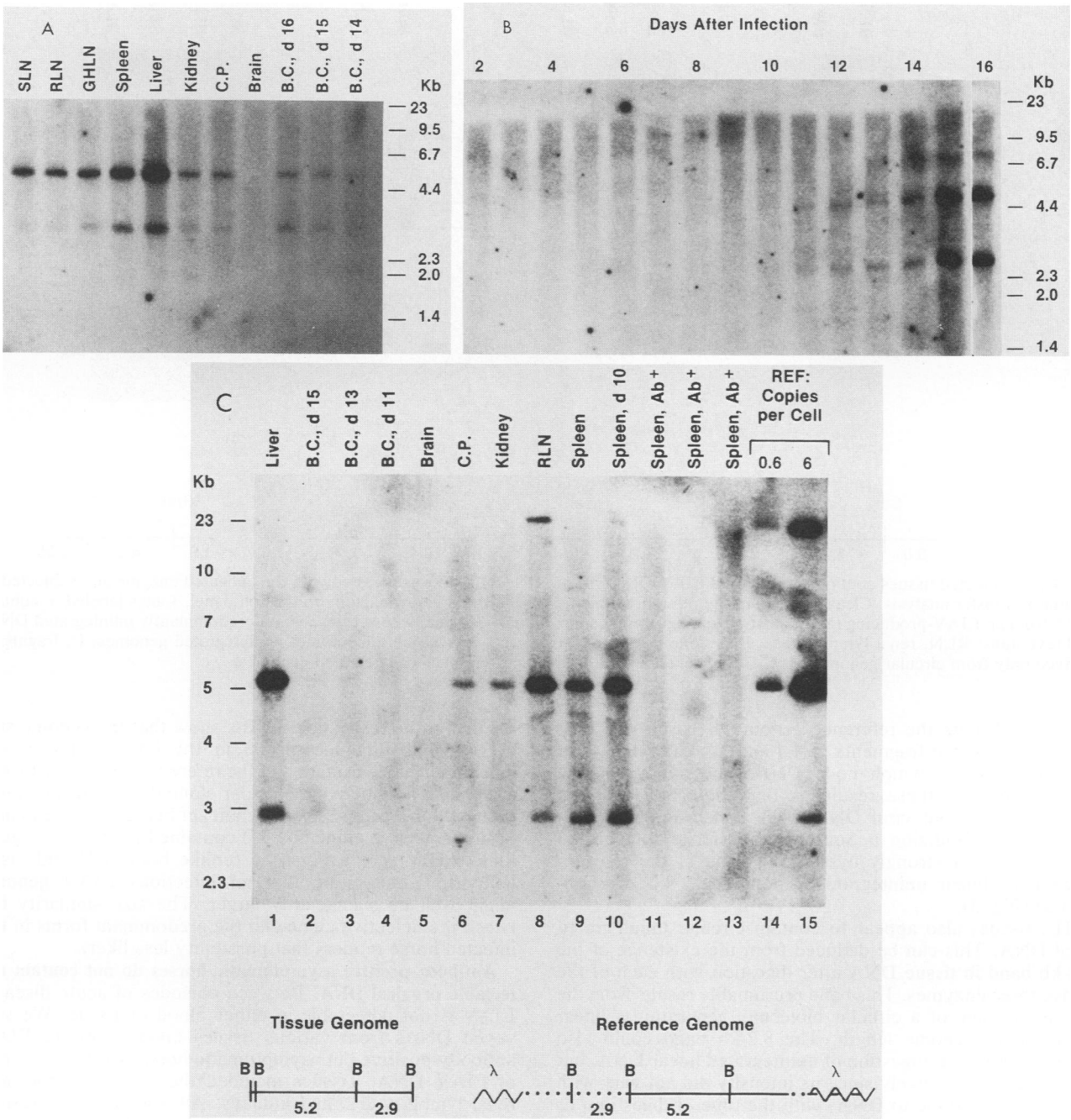

FIG. 2. EIAV DNA in tissues of an infected horse. (A) Southern transfer of DNA from tissues at 16 days p.i. and from circulating leukocytes at days 14, 15, and 16 p.i. Each lane contained about 5 μg of DNA which had been digested with *Bam*HI. The filter was exposed to X-ray film for 3 days at −70°C with an intensifying screen. Abbreviations: SLN, splenic lymph nodes; RLN, renal lymph nodes; GHLN, gastrohepatic lymph nodes; C.P., choroid plexus; B.C., d16, buffy coat, day 16 p.i. The sizes (in kilobases) of DNA standards are shown on the right. (B) DNA from daily buffy coat preparations. Each lane contained about 5 μg of a *Bam*HI digest. The film was deliberately overexposed to enable detection of bands in the samples taken on days 11 through 13. (C) Quantitation of EIAV proviral DNA in infected tissue. Samples (10 μg) of the indicated tissue DNA were digested with *Bam*HI and subjected to Southern transfer analysis. The filter was exposed to X-ray film for 3 days at −70°C with an intensifying screen. Lanes 14 and 15 contain DNA from a molecular clone of EIAV (25) in amounts equivalent to 0.6 and 6 copies per cell for 10 μg of mammalian cellular DNA. Hybridizing fragments of this clone and of the tissue samples arise as shown in the diagrams; ——, viral genome; ····, cellular flanking DNA; ∿∿∿, λ arms. Except where noted, tissue samples are from an acutely infected horse 16 days p.i. Abbreviations: B.C., d 15, buffy coat at day 15; C.P., choroid plexus; spleen, d 10, spleen from an acutely infected horse 10 days p.i.; spleen, Ab^+, spleens from horses with circulating antibody to EIAV p26. The faint band at about 7 kb in lane 12 was not reproducible.

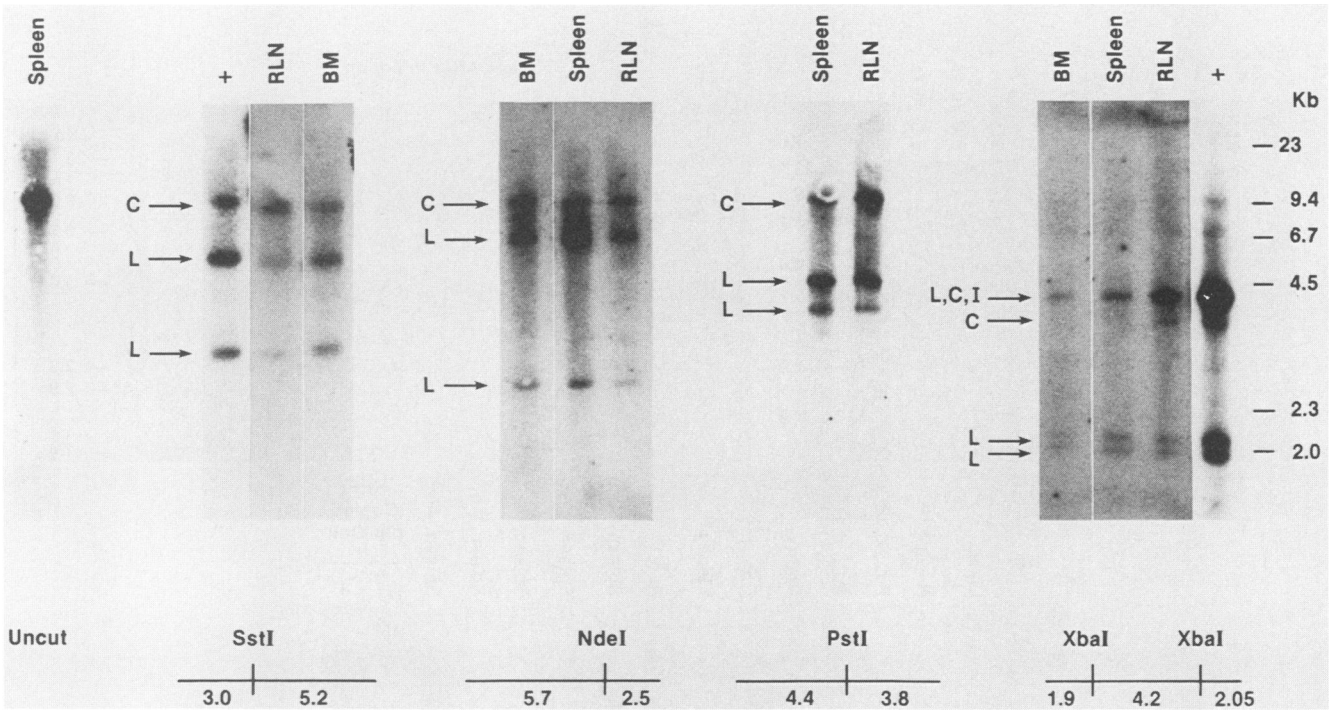

FIG. 3. Infected tissues contain unintegrated EIAV DNA. Tissue DNA (10 μg) was digested with the indicated enzyme and subjected to Southern transfer analysis. Cleavage sites in the reference genome are indicated below each hybridization panel. Lanes labeled + contain DNA from an EIAV-producing (Malmquist-Wyoming strain) canine thymus cell line, whose cells contain predominantly unintegrated DNA. Abbreviations: RLN, renal lymph nodes; BM, bone marrow; L, fragments derived only from linear unintegrated genomes; C, fragments derived only from circular genomes; L,C,I, fragments derived from linear, circular, or integrated genomes.

*Pst*I, which cuts the reference genome at base 4436, also gave the expected fragments of 4.4 and 3.8 kb in digests of spleen and renal lymph node DNA. The most probable conclusion from these results is that the tissues contain linear unintegrated viral DNA. This conclusion was confirmed by hybridizing a Southern transfer of undigested spleen DNA; a strongly hybridizing band at the size expected for linear unintegrated genomes (8.2 kb) was observed (Fig. 3).

The tissues also appear to contain circular unintegrated viral DNA. This can be deduced from the existence of the 8.2-kb band in tissue DNA after digestion with each of the above three enzymes. This band presumably results from the single cleavage of a circular molecule, generating a linear molecule of genome length. The 8.2-kb band could also result from partial digestion of unintegrated linear DNA, but we think this is unlikely since its intensity did not vary with the ratio of enzyme to DNA, with the time of digestion, or with the DNA preparation.

Digestion with *Xba*I, which cuts the reference genome twice (at bases 1913 and 6142), confirmed the above conclusions. A linear unintegrated molecule should generate fragments of 1.9, 4.2, and 2.05 kb, all of which were observed in DNA from the spleen, renal lymph nodes, and bone marrow. A circular molecule containing two long terminal repeats should generate, in addition to the 4.2-kb internal fragment, a 3.95-kb fragment; this was observed in the renal lymph node DNA but only weakly in the bone marrow and spleen DNA. Integrated DNA should also yield the internal 4.2-kb fragment. The face that the intensities of the 4.2-, 2.05-, and 1.9-kb bands are similar indicates that most (but presumably not all) of the EIAV DNA in these cells is unintegrated.

Finally, the restriction digests show that the genome size of the predominant virus(es) in the infected horse was indistinguishable from that of the reference genome, which is a replication-defective molecular clone derived from equine tissue culture cells. This is significant because the sequence of that defective clone (24, 25) contained neither a *nef* gene (unlike HIV) nor a *vip* gene (unlike both HIV and visna lentivirus), suggesting that an infectious EIAV genome might well be significantly larger. The size similarity between the defective clone and the predominant forms in the infected horse renders that possibility less likely.

Antibody-positive asymptomatic horses do not contain detectable proviral DNA. Between episodes of acute disease, EIAV is not detectable in either blood or tissue. We surveyed DNAs from various tissues taken from six EIAV antibody-positive but asymptomatic horses for the presence of EIAV DNA. Tissues included the spleen, splenic and renal lymph nodes, and kidneys. All Southern blot results were negative, with a level of sensitivity of about 0.05 copy per cell (Fig. 2C). Since blood and tissues from horses with chronic disease are infectious, we assume that they contain EIAV DNA. However, if the latent genomes are present in low copy number in a very small percentage of cells, a much more sensitive technique (e.g., the polymerase chain reaction) will be required to reveal them.

Target cell. Part of the evidence for the hypothesis that EIAV replication occurs predominantly in monocytes or macrophages or both is the observation that field strains of the virus can be propagated only in horse leukocyte cultures (14, 17). We have extended previous studies by surveying 10 additional cell lines for susceptibility to infection by the Wyoming strain. We applied 10^4 to 10^5 horse leukocyte

infectious doses to 2×10^5 cells in the presence of DEAE-dextran (20 μg/ml) and tested culture fluid for virus by immunoblotting at 1, 4, and 12 weeks p.i. There was no evidence of productive infection in any of the cell types tested, including the Cf2th canine thymus line, the Fea feline line, and several equine dermal cell lines and cell cultures derived from fetal equine liver, skin, heart, spleen, and ovaries. In contrast, horse leukocyte cultures were readily infected. Viral replication in buffy coat preparations selected for adherent cells was monitored by immunoblotting for EIAV p26 and by the occurrence of a marked cytopathic effect (data not shown).

These infectivity tests are thus completely consistent with the results of the earlier work, in which viral replication was observed only in horse leukocyte cultures. It is not known how many of the different cell types found in a typical buffy coat preparation can be infected with EIAV, but it can be said with certainty that the adherent cells are susceptible (7, 14, 26, 27). These cells are almost exclusively mononuclear (15) and hence presumably are of the monocyte-macrophage lineage. Cells of this lineage have also been implicated as targets for EIAV by immunofluorescence studies of acutely infected tissue (21). Fluorescing cells were found in many organs, including the spleen, liver, lymph nodes, kidney, thymus, and bone marrow; by the criteria of morphology and distribution, these cells appeared to be at least predominantly macrophages. On the grosser level of distribution of viral DNA, the results presented here are consistent with a monocyte-macrophage target. If we estimate the total number of macrophages per liver and the total number of circulating monocytes, and if we assume that macrophages and monocytes are equally susceptible to infection, we should be able to predict the relative amount of EIAV DNA in the liver versus buffy coat. The following assumptions are made: (i) 10% of total liver cells are Kupffer cells (12); (ii) cells make up 80% of the volume of the liver (2); (iii) the volume of an average liver cell is about 3,300 μm^3; (iv) the liver of a 500-kg horse weighs 7.4 kg; (v) the total blood volume of a 500-kg horse is 53 liters; and (vi) on day 16 p.i. there were 100 monocytes per μl of blood. The result is that there are about 1.8×10^{11} macrophages per liver and about 5.3×10^9 circulating monocytes, a 34-fold difference in favor of the Kupffer cells. Given the uncertainties in the above assumptions, this difference corresponds reasonably well to the observed distribution of EIAV DNA on day 16 p.i.: there was almost 90-fold more EIAV in the total liver than in the total buffy coat. These results are therefore consistent with a predominantly monocyte-macrophage target for EIAV.

In summary, we established an acute infection with a virulent field strain of EIAV and determined the amounts and distribution of viral DNA in blood and in various tissues. We detected the highest concentrations of viral DNA in the liver, lymph nodes, bone marrow, and spleen; however, the kidney, choroid plexus, and buffy coat DNAs were also positive. Brain DNA was negative (<0.05 genome per cell), but since others have found viral antigen-containing cells in the cerebrum and cerebellum (21), our negative result may be due to insufficient sensitivity. We found that the great majority of EIAV DNA is located in the tissues rather than in peripheral blood and that most of it exists in an unintegrated state, both linear and circular. Assuming that the macrophage is the principal target, each infected cell has multiple copies of viral DNA. Strategies utilizing this information are currently being employed to obtain molecular clones of EIAV from various tissues and, where possible, at various times after infection.

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