Equine Infectious Anemia Virus Replication Is Upregulated during Differentiation of Blood Monocytes from Acutely Infected Horses

DEBRA C. SELLON,^{1*} KATHY M. WALKER,¹ KAREN E. RUSSELL,¹ STEPHANIE T. PERRY,²
PATRICE COVINGTON,¹ AND FREDERICK J. FULLER²

*Departments of Food Animal and Equine Medicine¹ and Microbiology, Pathology, and Parasitology,²
North Carolina State University College of Veterinary Medicine, Raleigh, North Carolina 27606*

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Equine infectious anemia virus is a lentivirus that replicates in mature tissue macrophages of horses. Ponies were infected with equine infectious anemia virus. During febrile episodes, proviral DNA was detectable, but viral mRNA was not detectable. As cultured blood monocytes from these ponies differentiated into macrophages, viral expression was upregulated. In situ hybridization confirmed that viral transcription occurred in mature macrophages.

Equine infectious anemia virus (EIAV) is a lentivirus of horses that may provide insights into the important but complex interactions between blood monocytes, tissue macrophages, and human retroviruses (11, 25). Unlike infection with most other lentiviruses, infection of horses with EIAV frequently results in acute febrile illness within 1 to 2 weeks and is followed by a syndrome of recrudescing fever, weight loss, ventral edema, and anemia (22). Each episode of viremia and fever is associated with the emergence of a novel antigenic variant of the virus and a high-titer viremia (11, 20). As with lentivirus infections in other species, once infected with EIAV, a horse remains infected for life. Although EIAV has been recognized as an important cause of disease in horses since the early 1900s, many steps in EIAV pathogenesis have not been clearly elucidated. Following infection with a virulent wild-type strain of EIAV, most horses become febrile and thrombocytopenic within 7 to 14 days (2, 22). This febrile episode, like those that follow, is associated with a high titer of viremia in which virus is predominantly cell free (5, 6). Plasma viremia subsides coincident with resolution of clinical signs. In asymptomatic animals, only a very low titer of viremia is present, and infectivity is cell associated (5, 6). During febrile periods, the virus replicates predominantly in mature tissue macrophages of the spleen, liver, lymph nodes, lung, kidney, and adrenal gland (23). Although viral DNA is detectable in peripheral blood mononuclear cells by PCR, no viral RNA is detectable by in situ hybridization of adherent cells collected from febrile horses (23).

The type of nucleated blood cell containing EIAV provirus in vivo has never been determined. Because blood monocytes are the precursors of tissue macrophages, the primary in vivo host cell for EIAV replication during acute infections, it is probable that blood monocytes harbor EIAV provirus in vivo. Maury developed an in vitro model to investigate the role of monocyte maturation in regulating EIAV expression (9). Non-adherent peripheral blood monocytes were infected in vitro with a macrophage-tropic strain of EIAV. A delay in viral expression was observed in nonadherent cells compared with cells that were allowed to adhere and differentiate.

Extrapolating from our previous studies (23) and those of Maury (9), we hypothesized that blood monocytes in horses acutely infected with a virulent strain of EIAV contain proviral DNA. When those monocytes differentiate into macrophages, viral transcription and replication are upregulated.

Experimental infections with WYO-WT EIAV. Four ponies were infected with EIAV stocks derived from the virulent wild-type Wyoming (WYO-WT) strain of EIAV (4). Complete blood counts and serum reverse transcriptase (RT) activity were evaluated prior to infection and on selected days postinfection (2). RT activity was determined after viral purification from 10-ml aliquots of serum. Serum was layered on 5 ml of 20% sucrose in TNE (25 mM Tris-HCl [pH 7.4], 0.15 M NaCl, 2 mM EDTA) and centrifuged at $100,000 \times g$ at 4°C for 1 h. The pellet was resuspended in 100 μ l of phosphate-buffered saline (PBS), and a 10- μ l aliquot of the solution was assayed for RT activity. Aliquots were mixed with 20 μ l of RT cocktail consisting of 50 mM Tris-HCl (pH 7.9), 50 mM KCl, 5 mM MgCl₂, 5 mM dithiothreitol, 0.05% Nonidet P-40, 0.33 A₂₆₀ unit of poly(rA)-poly(dT)_{12–18} per μ l, 10 mM EGTA, and 0.5 mM [³H]TTP (62 Ci/mmol). Reaction mixtures were incubated for 90 min at 37°C, and 30 μ l of each sample was spotted on Whatman DE81 filters. Filters were washed three times in 0.35 M Na₂HPO₄, rinsed briefly in distilled H₂O, and washed once in 95% ethanol. Filters were air dried and counted in liquid scintillation-counting cocktail (Ecoscint O; National Diagnostics, Atlanta, Ga.) with an automated beta counter.

All horses had a rectal temperature $>39.5^{\circ}\text{C}$ within 14 days of infection with WYO-WT EIAV. There was a temporal correlation between changes in rectal temperature and changes in serum RT activity, with peak serum RT activity ranging from 26,730 to 84,892 cpm/ μ l. Increasing temperature and serum RT activity were associated with decreasing erythrocyte numbers and decreasing platelet counts in all ponies.

Circulating buffy coat cells contain EIAV proviral DNA. Rice et al. (18) and O'Rourke et al. (13) have previously demonstrated that buffy coat cells of acutely infected horses contain proviral DNA. We confirmed this observation in our infected ponies. Buffy coat cells were collected from each pony preinfection and at the indicated days postinfection when the rectal temperature had increased to $>39.4^{\circ}\text{C}$. Leukocyte-enriched plasma (buffy coat cells) was harvested from EDTA-anticoagulated blood after centrifugation at $120 \times g$ for 3 min

* Corresponding author. Mailing address: College of Veterinary Medicine, North Carolina State University, 4700 Hillsborough St., Raleigh, NC 27606. Phone: (919) 515-4479. Fax: (919) 515-3044.

FIG. 1. Southern blot of PCR-amplified fragments of EIAV proviral DNA. DNA was extracted from peripheral blood buffy coat cells as described in the text. DNA samples were amplified with primers spanning a 382-bp segment of the EIAV *pol* gene. Lanes: A, negative reagent control (no DNA added); B, pony 1, preinfection; C, pony 2, preinfection; D, pony 3, preinfection; E, pony 1, postinfection day 13; F, pony 2, postinfection day 9; G, pony 3, postinfection day 15; H, positive control amplification product.

to pellet erythrocytes. Aliquots of buffy coat cells were frozen at -70°C until assayed. This preparation technique results in buffy coat consisting of granulocytes, lymphocytes, monocytes, and platelets. After thawing, residual erythrocytes were lysed and DNA was extracted from nucleated buffy coat cells (granulocytes, lymphocytes, and monocytes) for amplification of EIAV proviral DNA (15, 27). Primers amplified a highly conserved 382-bp segment of the EIAV *pol* gene. Specificity of the amplification product was verified by Southern blotting using a ^{32}P -labeled nick-translated probe of pER, an avirulent molecular clone of EIAV (15). Results are shown in Fig. 1.

At the time of the initial viremia, all four ponies had detectable EIAV proviral DNA in their buffy coat cells. Other investigators have similarly demonstrated the presence of proviral DNA in equine peripheral blood cells during acute viremic episodes (13, 18). While we made no attempt to quantitate the amount of provirus in these ponies, Rice et al. have shown that during the initial viremic episode, circulating buffy coat cells contain about 10-fold less proviral DNA than does liver (18).

We have previously shown that viral mRNA is not detectable by *in situ* hybridization in any buffy coat cells (23). This indicates that although EIAV provirus is present in some subpopulation of these cells (granulocytes, lymphocytes, and monocytes), very little viral transcription is occurring. Peripheral blood monocytes are the precursors of tissue macrophages, the primary cellular sites of EIAV replication during acute viremic episodes. It is probable that circulating monocytes are the source of the proviral DNA that we and other investigators have detected in buffy coat cells from acutely infected horses.

After migration from blood into tissues such as the liver, bone marrow, spleen, and lymph nodes, monocytes mature and differentiate into macrophages. EIAV may directly infect fully differentiated macrophages, and/or it may infect undifferentiated monocytes and remain transcriptionally silent until signals are received as the cell undergoes differentiation in appropriate tissues. Maury presented evidence supporting the latter hypothesis with an *in vitro* model of macrophage differentiation and viral expression (9). We hypothesized that EIAV provirus was present in the monocyte fraction of buffy coat cells from acutely infected ponies and that transcription would be upregulated if those cells were allowed to differentiate *in vitro* by adherence to plastic.

EIAV replication is upregulated in adherent peripheral blood mononuclear cell cultures from acutely infected ponies. On the indicated days postinfection, blood was collected from each pony for isolation of monocyte-derived macrophages for culture. Equine adherent macrophage cultures were established by separation of peripheral blood mononuclear cells with Ficoll 1.077 (15, 27). Peripheral blood mononuclear cells were washed four times with PBS and plated in 12-well tissue culture plates with RPMI plus 10% normal horse serum. After 24 h, nonadherent cells were removed by three washes with PBS. Aliquots of supernatant were collected for RT assay every other day following culture. We have previously shown that by days 5 to 6 of culture, in this culture system, $>95\%$ of cells are monocyte-derived macrophages. Cells are nonspecific esterase positive and stain with a monoclonal antibody specific for a cytoplasmic antigen of equine mononuclear phagocytes (data not shown) (21).

In most experiments, mononuclear cells from 10 ml of whole blood were plated in each well of a 12-well plate. This resulted in approximately 10^7 mononuclear cells per well; the number of macrophages remaining in culture at day 7 of culture was approximately 0.5×10^6 to 1×10^6 per well, with 95% viability. The absolute number of viable cells differed between cultures from horses obtained at separate time points. Because of this inherent variability in the number of macrophages plated in each experiment, RT activities are directly compared only between wells of macrophages from the same horse plated at the same time and maintained under the same conditions. Typically, an *in vitro* infection of these monocyte-derived macrophage cultures with 10,000 cpm (RT activity) of WYO-WT EIAV would result in peak supernatant RT activity (usually obtained at postinfection days 7 to 9) ranging from 80,000 to 200,000 cpm/ μl . *In situ* hybridization of these cells reveals that 100% of the cells in the culture are infected and producing EIAV mRNA under these conditions (data not shown).

To rule out *de novo* infection of monocyte-derived macrophages *in vitro*, we sought to verify the absence of cell-free EIAV in the washed mononuclear cell preparation. Aliquots of the last macrophage wash solution were added to equine adherent macrophage cultures established from an uninfected, seronegative horse. Supernatant aliquots were collected every other day and assayed for RT activity as described above. No RT activity was detectable in the uninfected macrophage cultures to which aliquots of the last wash solution were added, verifying the absence of cell-free virus in the washed mononuclear cell preparations. In contrast, adherent blood mononuclear cell cultures rapidly developed detectable supernatant RT activity that increased over time (Fig. 2). These results demonstrate that peripheral blood cells previously infected *in vivo* are the source of supernatant RT activity measured in these experiments. Cultured macrophages from infected horses produced peak supernatant RT activities of 4,000 to 11,000 cpm/ μl (Fig. 2). This result is consistent with a low-multiplicity infection.

EIAV replication occurs in peripheral blood monocyte-derived macrophages. To identify the cell type in which EIAV was replicating and verify the establishment of a low-multiplicity infection, we used *in situ* hybridization to identify EIAV mRNA transcripts in specific cells. We collected peripheral blood mononuclear cells from an acutely infected pony as described above and cultured the adherent cell population in double-chamber slides. At various times postculture, cells were washed, the chamber was removed, and slides were fixed in 4% paraformaldehyde. After dehydration through graded ethanol, slides were frozen at -70°C until assayed. *In situ* hybridization was performed as previously described (23). After denatur-

FIG. 2. Supernatant RT activity from macrophage cultures established from ponies acutely infected with WYO-WT. Monocytes were purified from peripheral blood as described in the text. An aliquot of the final wash solution from the purification procedure was added to uninfected primary equine macrophage cultures. No supernatant RT activity was detectable in the cultures to which wash solution had been added, confirming the efficacy of the wash procedure in removing residual cell-free virus from the monocyte preparations. Symbols: ▼, pony 1, macrophages; ◆, pony 2, macrophages; ●, pony 3, macrophages; ■, pony 1, wash; ▲, pony 2, wash; ▲, pony 3, wash.

ation with 0.2 M HCl at room temperature and $2\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 70°C , cells were refixed briefly in fresh 4% paraformaldehyde and rinsed with PBS. Nonspecific binding was blocked with 0.01 M *N*-ethylmaleimide–0.01 M iodoacetamide in PBS followed by 0.25% acetic anhydride in 0.1 M triethanolamine buffer. After dehydration through graded ethanol, each slide was hybridized with 50,000 cpm of the appropriate probe in 5 μl of hybridization mix under a siliconized coverslip. One chamber of each slide was hybridized with a message antisense probe, and the other chamber was hybridized with a message sense probe (negative control). Slides were immersed overnight in mineral oil at 45°C . Control samples included equine dermal cells infected with a cell-adapted strain of EIAV and uninfected equine macrophages.

^{35}S -labeled riboprobes were prepared as previously described from a plasmid containing an 825-bp fragment from the *gag* gene of EIAV (23). The percent incorporation of [α - ^{35}S]UTP into the final probe was typically 70 to 80% (specific activity, approximately $10^9/\text{mg}$). The positive probe was complementary to the viral message and 280 bases in length. The negative probe was message sense and 545 bases in length.

After hybridization, slides were rinsed in chloroform twice to remove residual mineral oil, and coverslips were floated off in $2\times$ SSC. Washing and RNase treatments to remove nonspecifically bound probe were performed as described previously (23). Slides were dehydrated through graded ethanol and dipped in undiluted Kodak NTB-2 emulsion. They were stored in a lighttight slide box at 4°C for 7 days. After development with Kodak developer and fixer, the slides were rinsed, dried, and routinely stained with a rapid Giemsa-type stain (Leuko-Stat; Fisher Scientific, Orangeburg, N.Y.).

As shown in Fig. 3A, in situ hybridization of freshly collected buffy coat revealed no silver grains over any cells. Similarly, in situ hybridization of adherent mononuclear cells harvested 24 h after culture revealed no silver grains over any cells in the culture (data not shown). Figure 3B shows results of in situ hybridization of adherent cell cultures from the same pony harvested at 8 days after culture. Viral mRNA is detectable in cells with typical mononuclear phagocyte morphology. The mean supernatant RT activity for these chamber slides was approximately 3,538 cpm/ μl .

Buffy coat cells from acutely infected ponies contain EIAV proviral DNA, indicating the presence of intracellular virus

(Fig. 1). However, these cells express no detectable viral *gag* mRNA immediately after collection or during the first 4 days of culture (Fig. 3A), indicating that the virus is not transcriptionally active. If monocytes from these buffy coat preparations are allowed to mature in vitro and differentiate into macrophages, viral mRNA becomes detectable by 6 to 8 days of culture (Fig. 3B). Although some cell-to-cell spread of the virus cannot be ruled out in these cultures, it is likely that the first cells to be infected (i.e., those infected in vivo) would be the first cells to express viral mRNA. At 6 to 8 days of culture, approximately 1 in 30 macrophages express viral mRNA detectable by in situ hybridization. If it is assumed that all monocytes that contain proviral DNA in vivo are capable of supporting viral transcription after the monocyte differentiates to a macrophage, it may be estimated that $<10\%$ of circulating monocytes in these acutely infected ponies contained proviral DNA.

As monocytes migrate from the blood into tissues and differentiate into mature tissue macrophages, numerous genetically controlled events occur. Differentiation of monocytes involves expression of several cellular oncogenes, some of which (*c-fos*, *c-myc*, and *c-myb*) are expressed early in differentiation and others of which (*c-fms*, *c-src*, and *c-sis*) are expressed later (17). These genes trigger numerous signal transduction mechanisms and transcriptional regulatory activities. It is possible that expression of normal cellular differentiation genes similarly activates viral transcriptional mechanisms through binding to specific sequences of the viral long terminal repeat.

There is no evidence that the final tissue destination of individual blood monocytes is genetically predetermined, yet macrophages in specific tissues exhibit clear morphologic, functional, and phenotypic differences (1). These may result from differences in the microenvironment of a specific tissue and the differentiation signals received by macrophages in that tissue. Similarly, alterations in tissue microenvironment and cellular differentiation signals may affect the transcriptional upregulation of EIAV in macrophages in that tissue. For example, tissue macrophages of the spleen, liver, lymph nodes, lungs, kidneys, and adrenal glands contain large numbers of viral transcripts during the acute febrile episode, while very few transcripts can be found in macrophages of other endocrine or reproductive organs (23).

Increase in viral expression as monocytes differentiate into macrophages has been demonstrated with other primarily monocytopathic lentiviruses, including caprine arthritis-encephalitis virus (12) and visna-maedi virus of sheep (3). In contrast, human immunodeficiency virus type 1 (HIV-1) infection may be more productive in undifferentiated monocytes than in mature macrophages (27). Purified peripheral blood CD4^{+} monocytes inoculated with HIV in suspension produced only low levels of virus even after the cells differentiated into macrophages (8). In differentiated macrophages, HIV-1 transcription occurs but transcripts accumulate intracellularly without substantial release of progeny virions from the cells (14, 24).

These observations may be related to the role of macrophages in the maintenance of lentivirus infections and in the pathogenesis of clinical signs. In EIAV infections, viremia and subsequent viral dissemination in the body depend largely on viral replication in tissue macrophages and release of progeny virions into circulation (23). A high-titer viremia is consistently associated with clinical signs of fever, depression, and thrombocytopenia. Between viremic episodes, virus transcription is presumably restricted in some subpopulation of tissue macrophages, largely as a result of the host immune response against specific viral variants (16). As a new antigenic variant emerges,

FIG. 3. In situ hybridization of macrophage. (A) Buffy coat cells were centrifuged onto glass slides and hybridized with a message antisense probe to detect EIAV mRNA from the *gag* gene. (B and C) Peripheral blood mononuclear cells from a febrile horse acutely infected with EIAV. Cells were allowed to adhere to glass slides for 7 days after collection and hybridized with a message antisense probe (B) or a message sense probe (C) as described in the text. The arrow indicates a macrophage expressing viral mRNA. (D) Peripheral blood macrophages from an uninfected horse incubated with final wash solutions as described in the text. Macrophages were hybridized with a message antisense probe. (E and F) Equine dermal cells infected with a cell-adapted strain of EIAV and hybridized with a message antisense (E) or message sense (F) probe.

viral transcription is upregulated and clinical signs recur until immune responses again terminate replication (19, 22). Once immune responses are sufficient, viremic episodes are no longer observed unless the horse is immunosuppressed (e.g., by stress or dexamethasone administration) (7).

In summary, peripheral blood nucleated cells collected from ponies during the initial febrile episode following infection with WYO-WT EIAV contain EIAV proviral DNA. Viral transcription and replication are upregulated as peripheral blood monocytes are induced to differentiate into macrophages by adherence to plastic. This may have important implications in understanding the control of viral replication *in vivo* and the maintenance of persistent infection. Identification of the specific cellular factor(s) involved in upregulation of viral transcription in differentiated macrophages may be important in developing strategies for virus control.

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