Endotoxin treatment of equine infectious anaemia virus-infected horse macrophage cultures decreases production of infectious virus

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Lentiviruses replicate in cells of the immune system, and activation of immune cells has been shown to modulate virus replication. To determine the effects of macrophage activation on replication of equine infectious anaemia virus (EIAV), primary horse macrophage cultures (HMCs) were established from 20 different horses, infected with an avirulent strain of EIAV, and stimulated with 5 µg/ml of bacterial endotoxin. Supernatants collected from HMCs were assayed for the presence of tumour necrosis factor (TNF-α) and for production of infectious virus. Results indicated that EIAV replication in vitro varied significantly (P < 0.0001) from horse to horse, regardless of the treatment of HMCs. Also, EIAV replication was significantly (P < 0.0001) decreased in HMCs stimulated with bacterial endotoxin as compared to untreated HMCs. No significant correlation was found between virus replication and production of TNF-α following treatment of virus-infected cells with bacterial endotoxin. However, when HMCs were treated with endotoxin prior to virus infection, inhibition of EIAV replication was proportional to increasing levels of endotoxin. PCR and RT-PCR were used to amplify EIAV proviral DNA and mRNA sequences, respectively, at various time-points following infection. The results indicated that the early events of EIAV replication, up to and including transcription of multiple-spliced mRNAs, were not inhibited by treatment of EIAV-infected macrophages with bacterial endotoxin. This suggests that endotoxin treatment inhibits a post-transcriptional step in the virus replication cycle.

Introduction

Equine infectious anaemia virus (EIAV) is a member of the lentivirus subfamily of retroviruses. Like other lentiviruses, EIAV has a complex genome organization and replicates in cells of the monocyte/macrophage lineage (Narayan & Clements, 1989; Selton et al., 1992). EIAV induces a variable disease course in infected horses, described as acute, chronic or subclinical, and both viral and host factors are thought to be important in determining the clinical outcome of EIAV infection. Viral factors include the virulence of different isolates, antigenic variation to escape host immune surveillance, and sequence heterogeneity in viral regulatory elements, as well as co-existence of varying genotypes (Alexandersen & Carpenter, 1991; Carpenter et al., 1991; Kemeny et al., 1971; Kono & Kobayashi, 1970; Kono et al., 1973; Maury et al., 1997; Salinovich et al., 1986). Host factors are less well defined, but probably include the age, immune competence and major histocompatibility complex haplotype of the horse (Perryman et al., 1988). In addition, qualitative differences in cellular cofactors that are involved in viral gene regulation may be contributing host factors. For example, variation in human immunodeficiency virus type 1 (HIV-1) replication has been shown to occur in macrophages isolated from different sites throughout the body, as well as among individual donors (Olafsson et al., 1991; Spira & Ho, 1995). This suggests that cellular differences, whether within an individual or among individuals, can have a significant impact on virus replication and may contribute to variability in the clinical manifestations of disease.

A variety of cellular factors have been shown to be important in the regulation of lentivirus replication. Activation of cells with phorbol esters or certain cytokines stimulate gene expression of HIV-1 (Chowdhury et al., 1990; Folks et al., 1987, 1989; Mellors et al., 1991; Poli et al., 1990; Israel et al., 1989).
simian immunodeficiency virus (Lairmore et al., 1991), EIAV (Carvalho & Derse, 1993), visna virus (Shih et al., 1992) and feline immunodeficiency virus (Sparger et al., 1992). Many of these effects are attributed to the activation of cellular transcription factors which directly or indirectly interact with enhancer sequences present in the viral long terminal repeat (LTR), resulting in increased viral gene expression.

Activation of cells has also been reported to inhibit lentivirus replication. HIV replication is inhibited by treatment of infected cells with a phorbol ester, interferon (IFN) and bacterial lipopolysaccharide (LPS) (Gendelman et al., 1990, 1992; Kornbluth et al., 1990; Mufson et al., 1992; Poli et al., 1989; Shirazi & Pitha, 1992). The overall effects of cellular activation on lentivirus replication depend, in part, on the cell type used for in vitro studies. For example, cellular activation with a phorbol ester (Mufson et al., 1992) or LPS (Kornbluth et al., 1989) inhibits HIV replication in primary human monocytes, but stimulates virus replication in chronically infected cell lines (Chowdhury et al., 1990; Pomerantz et al., 1990). Activation of primary human macrophages with IFN has been found to inhibit early stages in HIV replication (Gendelman et al., 1990; Kornbluth et al., 1990; Meylan et al., 1993); in contrast, activation of cell lines is thought to inhibit late stages in the virus life-cycle (Gendelman et al., 1990; Poli et al., 1989).

It has been shown that the EIAV LTR can be transcriptionally activated by treating transiently transfected HeLa cells with a phorbol ester, and that activation is mediated through an ets/PEA3 motif present in the LTR (Carvalho & Derse, 1993). However, phorbol ester stimulation of primary equine macrophages was shown to decrease production of EIAV reverse transcriptase activity (Sellon et al., 1996). To characterize the cellular factors important in regulation of EIAV replication, we examined the effect of macrophage variability and activation on EIAV replication in primary horse macrophage cultures (HMCs) in vitro.

**Methods**

- **Horses.** Twenty horses from the Iowa State University Animal Science Department were used to examine the effect of the donor horse and macrophage activation on permissiveness for virus replication in vitro. Sixteen of the 20 horses were Quarterhorses and the other four were Arabian. The horses ranged in age from 2 to 23 years; four were mares and 16 were geldings. Sera from each animal were screened for antibody titres to equine herpesvirus (EHV) 1, 2, 3 and 4 (University of Kentucky Livestock Disease Diagnostic Center, Lexington, KY) and EIAV (Iowa State University Diagnostic Laboratory, Ames, IA). AntieHV-1, -2 and -4 antibodies were detected in samples from all horses; however, all samples were negative for EHV-3 and EIAV.

  An additional donor horse housed at the Iowa State University Veterinary Teaching Hospital was used to determine the mechanism of inhibition of EIAV replication in equine macrophages activated with bacterial endotoxin.

- **Cells.** Whole blood was obtained by jugular venipuncture or plasmapheresis. Mononuclear leukocytes were banded by centrifugation at 750 g on a discontinuous Histopaque gradient (Carpenter & Chesebro, 1989), resuspended in Dulbecco’s minimum essential medium (DMEM) containing 20% horse serum and 10% newborn calf serum (NCS), and seeded at 2 x 10^5 cells/cm^2 in 24-well tissue culture plates. Macrophages were allowed to adhere overnight at 37 °C and non-adherent cells were removed from the cultures by washing twice with Hank’s buffered saline solution (HBSS) containing 2% foetal calf serum (FCS). Cells were maintained in DMEM containing 20% horse serum, 10% NCS and antibiotics.

  Equine dermal (ED) cells (ATCC CCL57) were grown in DMEM supplemented with 20% FCS and antibiotics.

- **Virus inoculation and macrophage activation.** For initial experiments, HMCs were treated at 48 h with 8 µg/ml polybrene and infected with 1-10 x 10^4 focus forming units (f.f.u.) of MA-1, an avirulent strain of EIAV (Carpenter & Chesebro, 1989). The next day, cells were washed with HBSS containing 2% FCS to remove cell-free virus. Supernatant samples were collected every other day for 6 days post-infection (p.i.), and tested for the presence of virus as described below.

  EIAV-infected or uninfected HMCs from each animal were treated with either fresh medium or medium containing 5 µg/ml Escherichia coli K235 endotoxin (Sigma). Supernatant samples were collected from all wells immediately prior to, and 4 hours following, HMC activation, and tested for the presence of tumour necrosis factor (TNF-α) as described below.

  In some experiments, HMCs were activated with bacterial endotoxin prior to virus inoculation. At 24 h, cells were washed with HBSS containing 2% FCS and treated with fresh medium containing 0, 0.2, 1 or 5 µg/ml endotoxin. Supernatant samples were collected from all wells immediately prior to, and 4 h following, HMC activation, and tested for the presence of TNF-α. At 48 h, HMCs from each treatment group were infected with 1 x 10^4 f.f.u. MA-1 in the presence of 8 µg/ml polybrene. The next day, cells were washed with HBSS containing 2% FCS to remove cell-free virus. HMC supernatant samples were collected at 6 days p.i., and tested for the presence of infectious virus as described below.

- **Assays for virus replication.** Culture supernatant samples were clarified by centrifugation at 2000 x g for 6 min, and infectious virus was quantified using a focal immunoassay (FIA) which employed a chromogenic method of detection rather than the fluorogenic method previously described (Carpenter & Chesebro, 1989). HMC supernatant samples were serially diluted 10-fold in DMEM containing polybrene (8 µg/ml) and added to cultures of ED cells. Five days post-inoculation, ED cells were washed once with TNE buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA pH 7.5), fixed with 100% methanol for 10 min, and washed again with TNE. The primary antibody, an EIAV convalescent horse serum (1:800), was added to the cells and allowed to bind for at least 1 h at room temperature. Unbound serum was removed by washing with three times with TNE and the cells were incubated for 30 min at room temperature with the secondary antibody, horseradish peroxidase-conjugated goat anti-horse IgG polyclonal antibody (1:800) (Organon Teknika). Unbound secondary antibody was removed as before, and AEC substrate (0.2 mg/ml 3-amino-9-ethylcarbazole with 0.01% hydrogen peroxide in 0.05 M sodium acetate solution pH 5.0), was added. The substrate was allowed to react for 10 min in the dark at room temperature, the cells were washed with tap water and allowed to dry at room temperature. Foci of infected cells were counted using a dissecting microscope.

- **TNF-α bioassay.** L929 cells (ATCC CCL1, NCTC Clone 929) were seeded at 4 x 10^4 cells per well in 90-well tissue culture plates in DMEM containing 10% horse serum and incubated for 24 h at 37 °C. HMC supernatant samples were diluted 1:5 and 1:50 in DMEM containing 3 µg/ml actinomycin D, and 100 µl of each dilution was added to duplicate wells of L929 cells. Cells were incubated overnight at 37 °C.
fixed with 50 µl 10% phosphate-buffered formalin, washed twice with 0.85% NaCl, and stained with a gravity-filtered solution of 0.2% crystal violet and 0.74% formaldehyde in distilled water. After washing the cells with tap water, plates were dried overnight at room temperature. The next day, 100 µl of a 1:1 solution of PBS–95% ethanol was added to each well of the plate, and the absorbance of the solution was read at 550 nm. A 10-fold serial dilutions of recombinant murine TNF-α (rTNF-α) (Genzyme) were added to duplicate wells of each test plate containing L929 cells. As negative controls medium alone, and 1 unit of EIAV-containing L929 cells. As negative controls medium alone, and 1 unit of rTNF-α were co-amplified as an internal control. β-actin-specific primers were designed based on sequences obtained from conserved regions of β-actin sequences (Table 1). All blots were stripped and probed with a β-actin-specific probe radiolabelled with 32P with random hexamers.

### Table 1. Oligonucleotide primers used for the amplification of viral and β-actin sequences

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence location</th>
<th>Primer sequence</th>
<th>Size of product</th>
<th>Amplified product</th>
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<tr>
<td>1a</td>
<td>1–20</td>
<td>TGTGGGCTTTTATGAGGG</td>
<td>320 bp</td>
<td>Early proviral DNA</td>
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<tr>
<td>1b</td>
<td>299–320</td>
<td>GTAGAATCTCAGACAGAAAAAC</td>
<td>506 bp</td>
<td>Late proviral DNA</td>
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<tr>
<td>2α‡</td>
<td>228–246</td>
<td>TGAATCCCTTCTCGTAG</td>
<td>429 bp</td>
<td>Unspliced RNA</td>
</tr>
<tr>
<td>2b</td>
<td>714–733</td>
<td>TGGAGGCCCATCTTACAGC</td>
<td>589 bp</td>
<td>Single-spliced RNA</td>
</tr>
<tr>
<td>3β</td>
<td>305–326</td>
<td>CTGGTCGAGATCCCTACAGTTG</td>
<td>359 bp</td>
<td>Triple-spliced RNA</td>
</tr>
<tr>
<td>3a</td>
<td>714–733</td>
<td>TGGAGGCCCATCTTACAGC</td>
<td>760 bp</td>
<td></td>
</tr>
<tr>
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<td>359 bp</td>
<td></td>
</tr>
<tr>
<td>5b</td>
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</tr>
<tr>
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<td>760 bp</td>
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</table>

* Oligonucleotide primers specific for EIAV were designed based on sequences obtained from GenBank (accession numbers M16575, M11337, K03334 and M14855), and used to amplify viral DNA and mRNA sequences by PCR and RT–PCR, respectively.
† Oligonucleotide primers specific for β-actin were provided by Michael Murtaugh at the University of Minnesota, and used to amplify β-actin DNA and mRNA sequences by PCR and RT–PCR, respectively.
‡ Base number 245, A, differs from the published sequence of G.
§ Primers were designed based on consensus sequences; therefore location is not applicable (NA).

### Isolation and amplification of proviral DNA. EIAV proviral sequences were amplified by PCR to determine the effect of macrophage activation on EIAV transcription. Total cellular DNA was isolated at 6, 18 and 43 h p.i., from infected and non-infected HMCs treated with each dilution of bacterial endotoxin. Cells lysates were prepared by SDS–proteinase K digestion, and DNA was extracted with phenol and chloroform–isoamyl alcohol and precipitated with ethanol. The amount of DNA used for each PCR reaction was normalized based on the number of cells from which each sample was originally isolated (4.5 × 10⁶ cells per reaction). EIAV early and late proviral transcripts were amplified using synthetic oligonucleotide primers based on published sequences (Table 1). Standard amplification conditions consisted of 35 cycles of primer annealing at 50 °C for 1 min, primer extension at 72 °C for 2 min, and denaturation at 94 °C for 1 min in an automated thermocycler (Perkin Elmer Cetus). Positive and negative controls used for each PCR reaction included a plasmid containing proviral MA-1 DNA, and distilled water. For analysis, 15 µl of the PCR products was electrophoresed in 2% agarose, transferred overnight to Hybond nylon membranes (Amersham) by an alkaline transfer technique, and hybridized with EIAV LTR-specific probes radiolabelled with 32P with random hexamers (Carpenter et al., 1991).

### Isolation and amplification of viral mRNAs. The effect of macrophage activation on EIAV transcription was determined using RT–PCR to amplify EIAV cDNA sequences from different viral mRNA species. HMCs were established, treated with bacterial endotoxin and infected with EIAV as described above. At 6, 18 and 43 h p.i., total cellular RNA was isolated from cultures using guanidinium thiocyanate (Stratagene) and phenol and chloroform–isoamyl alcohol extraction. RNA was precipitated from each sample with ethanol and quantified using a commercially available kit (Invitrogen). EIAV mRNA was reverse transcribed into DNA with random hexamer primers, and the cDNA was amplified by PCR using splice-specific primers (Table 1) which were designed based on published sequences (Kawakami et al., 1987). The amount of RNA used for each RT–PCR reaction was normalized according to the number of cells from which the RNA was originally isolated (9 × 10⁶ cells per reaction). Following amplification, 15 µl of the RT–PCR reaction products was electrophoresed in 2% agarose, transferred overnight to nylon membranes, and hybridized to probes specific for each corresponding amplified sequence. To ensure that comparable amounts of input RNA were used in each experiment, β-actin sequences were co-amplified as an internal control. β-actin-specific primers were designed based on regions obtained from conserved regions of β-actin DNA, and were kindly provided by Michael Murtaugh, University of Minnesota, USA (Table 1). All blots were stripped and probed with a β-actin-specific probe radiolabelled with 32P using random hexamer primers.

### Statistical analysis. Comparison of virus replication among horses and treatments was carried out using analysis of variance to determine the statistical significance of differences between the means of the samples. To examine the relationship between TNF-α production and
Results

EIAV replication varied among horses

The MA-1 isolate of EIAV was derived from a horse experimentally inoculated with a field isolate of EIAV and selected for in vitro replication on equine dermal cells (Carpenter & Chesebro, 1989). Previously, it was reported that MA-1 did not replicate to detectable levels in primary HMCs (Carpenter & Chesebro, 1989), and studies were initiated to define the basis for restriction of MA-1 replication in these cells. Preliminary experiments comparing MA-1 replication in HMCs derived from two different horses suggested that levels of MA-1 replication may vary with respect to the donor horse (data not shown). In the present study, we examined the influence of the donor horse and macrophage activation on MA-1 replication in HMCs in vitro.

Blood was collected from each of 20 horses on three separate occasions, and cells were infected with MA-1. Data obtained from all horses showed that MA-1 replication in untreated HMCs varied significantly (P ≤ 0.0001) among the 20 different horses (Fig. 1). In addition, virus replication in HMCs obtained from a single horse was highly variable with some horses (nos 5 and 9), and less variable with others (nos 3, 7, 11 and 20). No correlation was observed between levels of EIAV replication in HMCs established from different horses and the age, sex or breed of the horse. In addition, no relationship was found between serological antibody titres to EHV and levels of EIAV replication. This suggested that there are intrinsic differences among horses at the cellular level which are involved in regulation of virus replication.

Macrophage activation decreased EIAV replication

To examine the effects of macrophage activation on EIAV replication, HMCs established from the group of horses described above were infected with MA-1, and stimulated 24 h later with 5 µg/ml bacterial endotoxin. In 19 out of 20 horses, MA-1 replication significantly (P ≤ 0.0001) decreased following HMC activation (Fig. 1). One horse, no. 5, showed essentially no change in permissiveness for MA-1 between treated and untreated HMCs. Interestingly, this horse produced only 10³ f.f.u./ml MA-1 from untreated HMCs, the lowest titre of all 20 horses.

EIAV replication and TNF-α production

To determine whether the decline in EIAV replication in endotoxin-treated HMCs was due to differences in the level of macrophage activation, TNF-α production was measured in HMC supernatant samples in eight horses. These eight were chosen because they best represented the data obtained from HMCs of all horses used for the experiments. Two horses each were selected in which relatively high (nos 1 and 7) and low (nos 8 and 9) levels of virus replication occurred in their HMCs in vitro. Three animals (nos 13, 15 and 19) were selected in which a large decrease in virus replication occurred when HMCs from these animals were treated with endotoxin. In addition, horse no. 5 was included as virus replication in HMCs established from this animal was unaffected by endotoxin treatment. All samples from HMCs treated with endotoxin contained significantly higher amounts of TNF-α than samples from untreated wells (data not shown), indicating that all cultures were activated by the methods used.

Replication of other lentiviruses has been shown to be affected by TNF-α (Folks et al., 1989; Kinter et al., 1990; Lairmore et al., 1991; Poli et al., 1990), so data were analysed to determine whether there was a correlation between TNF-α production and virus replication in HMCs established from the same animal. No significant correlation was observed between levels of TNF-α produced and levels of virus replication in activated HMCs (Fig. 2A). Also, there was no significant difference between levels of TNF-α produced by MA-1-infected and uninfected cells, indicating that virus replication had no significant effect on TNF-α production in activated HMCs (data not shown). However, there was a greater than 10-fold decrease in MA-1 replication when detectable levels of TNF-α were observed at the time of infection, prior to activation of HMCs (Fig. 2B). This suggested that the activation state of the cell at the time of infection, as measured by TNF-α, may influence permissiveness for EIAV replication in HMCs.

To further examine the effects of endotoxin treatment on virus replication in HMCs activated prior to infection, HMCs were established from a donor pony, treated with serial dilutions of bacterial endotoxin at 24 h, and infected with EIAV at 48 h. HMC supernatant samples were collected and assayed...
for levels of TNF-α and titres of infectious virus. As suggested from the previous results, a dose-dependent relationship was observed between endotoxin treatment and EIAV replication: HMCs treated with high levels of endotoxin were less permissive for EIAV replication than HMCs treated with lower levels of endotoxin (Fig. 2C). In addition, HMCs treated with high levels of endotoxin produced more TNF-α than HMCs treated with less endotoxin. The high levels of TNF-α following endotoxin treatment indicated that the inhibition of virus replication was not due to a general decrease in cellular protein synthesis. There was no evidence of a direct correlation between TNF-α and EIAV replication, thus it does not appear that TNF-α is directly inhibiting virus replication.

**Effects of endotoxin treatment on EIAV reverse transcription**

Results from the above experiments suggested that cellular events associated with macrophage activation may restrict virus replication early after infection. Therefore we examined the effects of endotoxin treatment on reverse transcription, an event which occurs early in the life-cycle of the virus. Due to the pattern of reverse transcription, early and late proviral transcripts (RT transcripts) can be analysed by Southern blot analysis after PCR amplification using specific oligonucleotide primers (Fig. 3A). Early RT transcripts of proviral DNA were observed 18 h p.i., while late RT transcripts could not be detected until 43 h p.i. (Fig. 3B). At early time-points after infection, no significant differences in either the quality or quantity of early or late proviral DNA were attributable to the dose of endotoxin treatment (Fig. 3B). These data indicate that reverse transcription and prior events in the virus life-cycle, such as virus binding, entry and uncoating, are not significantly affected by treatment of HMCs with bacterial endotoxin.

**Detection of EIAV mRNA transcripts in endotoxin-activated HMCs**

To examine the effects of endotoxin treatment on the transcription of EIAV mRNAs, RNA was isolated from infected and control HMCs and analysed by Northern blot and RT–PCR. Results of the Northern blot analysis were inconclusive, due to the insufficient quantity of viral mRNA at early time-points following infection. Synthetic oligonucleotide primers (Table 1) designed to amplify splice-specific sequences from each viral mRNA (Fig. 4) were used in RT–PCR. The sensitivity and specificity of each primer pair were determined by amplification of control RNA isolated from MA-1 infected equine dermal cells. All of the primer pairs used for these experiments had similar levels of sensitivity and produced bands of the expected sizes after RT–PCR with the control RNA (data not shown). Co-amplification of β-actin sequences by RT–PCR was done to ensure that comparable amounts of RNA were present for analysis.
Endotoxin treatment had no quantitative effect on the level of triple-spliced mRNA transcripts at 43 h p.i. (Fig. 5). Similar results were observed using a different primer pair to detect the three-exon, double-spliced mRNA (data not shown). These results indicated that inhibition of EIAV replication at early time-points following infection was not associated with quantitative changes of multiply spliced mRNAs. Single-spliced mRNA transcripts were not detectable in either endotoxin-treated or untreated HMC RNA samples following 35 cycles of PCR amplification. However, when the PCR was extended to 45 cycles with primer pair 5, single-spliced mRNA was detected at 43 h p.i. from untreated HMCs, but remained undetectable in RNA from endotoxin-treated HMCs (Fig. 5). For increased sensitivity in the detection of single-spliced transcripts, nested PCR was performed using primer pairs 5a and 5c for the first reaction, followed by primer pair 5a and 5b. Using this method the presence of single-spliced mRNA was clearly detectable in untreated cells but only barely perceptible in cells treated with 1 or 5 µg of endotoxin (Fig. 5). By 6 days p.i., following multiple rounds of virus replication, all species of viral mRNAs were detected in both endotoxin-treated and control HMCs; however, the level of mRNAs decreased with
increasing doses of endotoxin (data not shown). Therefore, the effect of endotoxin treatment occurred at a post-transcriptional step in virus replication and may be associated with a delayed appearance of incompletely spliced mRNAs.

Discussion

Horse macrophage cultures established from 20 different horses varied significantly (P < 0.0001) in permissiveness for EIAV replication in vitro. The differences were not correlated with age, breed, sex or previous medical history. Individual environmental conditions present at the time of blood collection were minimized by sampling each horse three times over a 4 month period. Together, these results suggest that there are intrinsic differences among horses at the genetic and/or cellular level which regulate virus replication. These cellular differences may play a crucial role in determining the permissiveness for EIAV among horses, and as a result contribute to the clinical outcome of infection.

Previously, others have found significant variation in susceptibility to HIV infection among primary human macrophages and human peripheral blood lymphocytes (Olafsson et al., 1991; Spira & Ho, 1995). These differences have been attributed to differences among individuals in susceptibility to HIV infection and/or variability in levels of in vivo activation. Individual differences in susceptibility to infection with macrophage-tropic strains of HIV-1 are associated with the presence or absence of the CCR5 chemokine receptor (Dean et al., 1996; Huang et al., 1996; Liu et al., 1996), the co-receptor for macrophage-tropic strains of HIV. The receptor for EIAV is not known; however it is possible that the differences in receptor concentration may contribute to the observed differences among horses in susceptibility to EIAV. In addition, the present studies utilized only a single, cell culture-adapted isolate of EIAV and it is possible that different results might be obtained with primary field isolates of EIAV. No correlation was observed between EIAV replication and levels of macrophage activation as measured by production of TNF-α following endotoxin treatment. However, the decrease in EIAV replication in stimulated macrophages as compared to untreated macrophages indicates that regulation of viral replication is controlled, in part, by cellular activation signals.

Activation of HMCs using bacterial endotoxin resulted in significantly (P < 0.0001) decreased replication of EIAV. Similar inhibition of virus replication following macrophage activation was found following treatment of EIAV-infected macrophages with phorbol myristate acetate (PMA) (Sellon et al., 1996) or HIV-infected monocytes with bacterial LPS (Kornbluth et al., 1989). Activated macrophages may exert indirect antiviral effects mediated by IFN. TNF-α, as well as bacterial endotoxin, is capable of inducing IFN production by macrophages. IFN treatment of HIV-infected primary human macrophages can affect early steps in the virus replication cycle, between adsorption and reverse transcription, resulting in reduced viral DNA synthesis (Gendelman et al., 1990a; Kornbluth et al., 1990; Meylan et al., 1993). Other studies have demonstrated that LPS treatment inhibits HIV replication at the level of transcription (Kornbluth et al., 1989). Results from our experiments indicate that the early events of EIAV replication, up to and including transcription of single-spliced mRNAs, are not inhibited by treatment of EIAV-infected macrophages with bacterial endotoxin. This is consistent with the previous findings of Sellon et al. (1996), who found no differences in inhibition of EIAV replication among molecularly cloned EIAVs with LTRs predicted to differ in transcriptional response to PMA activation. Interestingly, our studies indicate that the predominant effect of endotoxin treatment on EIAV replication occurred post-transcriptionally and appeared to be associated with a delayed appearance of single-spliced mRNA.

The appearance of incompletely spliced viral mRNAs and production of infectious virus is dependent on the viral protein Rev. Therefore, it is possible that endotoxin treatment inhibits virus replication through direct, or indirect, inhibition of Rev function. Similar to HIV Rev, EIAV Rev mediates nuclear export of incompletely spliced viral mRNAs through interaction with a cellular nucleoprotein (Bogerd et al., 1995; Meyer et al., 1996). Additionally, EIAV Rev plays a role in splice-site selection, possibly through interaction with the splicing factor SF2/ASF (Gontarek & Derse, 1996). SF2/ASF is a member of a family of serine–arginine rich (SR) proteins that function as essential splicing factors and as regulators of alternative splicing (Fu, 1995; Manley & Tacke, 1996).

Fig. 5. Effects of bacterial endotoxin treatment on the transcription of EIAV mRNAs. HMCs established from a donor pony were treated with serially diluted bacterial endotoxin (0, 0.2, 1 and 5 µg/ml) at 24 h, and infected with EIAV at 48 h. Total cellular RNA was isolated at 43 h p.i. Primer pairs 5 and 9 were used to amplify single- and triple-spliced mRNAs (see Fig. 4). Nested RT–PCR amplification of single-spliced mRNA was performed using primers 5a and 5c for the first RT–PCR, followed by primers 5a and 5b for the second amplification. β-actin mRNA sequences were co-amplified in each RT–PCR reaction using specific primers (see Table 1). Reaction products were electrophoresed in 2% agarose, transferred to nylon, and hybridized with EIAV-specific probes. Blots were stripped and rehybridized with a β-actin-specific probe.
Interaction of SF2/ASF with an exon enhancer element in the bicistronic EIAV Tat/Rev mRNA is thought to promote exon skipping and down regulation of EIAV Rev in a Rev-dependent manner (Gontarek & Derse, 1996). More recently, Powell et al. (1997) reported Rev-dependent binding of SF2/ASF to the HIV Rev-responsive element. Interestingly, overexpression of SF2/ASF resulted in a dose-dependent inhibition of Rev function and down-regulation of HIV replication (Powell et al., 1997). Expression of SR proteins varies among tissues and cells at different stages of activation/differentiation (Zahler et al., 1993; Fu, 1995) and it has been shown that activation of T cells with phytohaemagglutinin increases expression of SRp30c (Screaton et al., 1995). At present it is not known whether endotoxin treatment of HMCs would alter expression of SF2/ASF or other SR proteins, or if altered levels of SR proteins would inhibit EIAV Rev function in stimulated macrophages. A better understanding of post-transcriptional regulation of viral gene expression may provide important insight into cellular factors which contribute to the dynamics of virus replication in vivo.

Another mechanism by which cellular factors induced by endotoxin could lead to post-transcriptional inhibition of EIAV replication is suggested by the work of Chisari and co-workers (Guidotti et al., 1996; Tsui et al., 1995). Their studies have shown that TNF-α and/or IFN-α/β mediate post-transcriptional inhibition of hepatitis B virus (HBV), and these investigators hypothesize that similar events may occur in other viral infections. It is possible that a cytokine-mediated intracellular antiviral process is activated by endotoxin treatment of HMCs. Elevated levels of TNF-α were found in endotoxin-stimulated HMCs, and Sellon et al. (1996) reported similar increases in TNF-α in PMA-stimulated macrophages. We observed no direct correlation between the level of TNF-α and virus titre, suggesting that TNF-α is not the direct or sole inhibitor of EIAV replication. However, cytokine-mediated inhibition of HBV appears to require both TNF-α and IFN-α/β (Guidotti et al., 1996). IFN levels were not evaluated in the present study, and additional studies are needed to elucidate the potential role of inflammatory cytokines in post-transcriptional regulation of EIAV replication.

We thank Dr Peggy Miller-Grabcr for her cooperation and assistance in the use of the Iowa State University horse herd. We thank Wendy Maury for her helpful discussions and Yvonne Wannemuehler, Mary Long, Sara Garr, Dawne Buhrow and Kurtimus Plagge for their excellent technical assistance. This work was supported in part by PHS grant AI30025.

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Received 8 September 1997; Accepted 27 November 1997