Complex Interactions Between the Major and Minor Envelope Proteins of Equine Arteritis Virus Determine its Tropism for Equine CD3⁺ T Lymphocytes and CD14⁺ Monocytes

Yun Young Go, Jianqiang Zhang, Peter J. Timoney, R. Frank Cook, David W. Horohov, and Udeni B. R. Balasuriya*

Maxwell H. Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, Kentucky 40546-0099, USA

* Corresponding author.

Mailing address: 108 Maxwell H. Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY 40546-0099.

Phone: 859-257-4757 ext. 81124; Fax: 859-257-8542; E-mail: ubalasuriya@uky.edu

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Abstract

Extensive cell culture passage of the virulent Bucyrus (VB) strain of equine arteritis virus (EAV) to produce the modified live virus (MLV) vaccine strain has altered its tropism for equine CD3+ T lymphocytes and CD14+ monocytes. The VB strain primarily infects CD14+ monocytes and a small subpopulation of CD3+ T lymphocytes (predominantly CD4+ T) as determined by dual-color flow cytometry. In contrast, the MLV vaccine strain has significantly reduced ability to infect CD14+ monocytes and has lost its capability to infect CD3+ T lymphocytes. Using a panel of five recombinant chimeric viruses, we demonstrated that interactions between GP2, GP3, GP4, GP5 and M envelope proteins play a major role in determining the CD14+ monocyte tropism while the tropism of CD3+ T lymphocytes is determined by GP2, GP4, GP5 and M envelope proteins but not the GP3 protein. The data clearly suggest that there are intricate interactions among these envelope proteins that affect binding of EAV to different cell receptors on CD3+ T lymphocytes and CD14+ monocytes. This study shows for the first time that CD3+ T lymphocytes may play an important role in the pathogenesis of equine viral arteritis when horses are infected with the virulent strains of EAV.
Introduction

Equine arteritis virus (EAV) is a small enveloped virus with a positive-sense, single-stranded RNA genome of 12.7 kb that belongs to the family Arteriviridae (genus Arterivirus, order Nidovirales), which also includes porcine reproductive and respiratory syndrome virus, simian hemorrhagic fever virus, and lactate dehydrogenase-elevating virus of mice (11, 55). The EAV genome includes nine known functional open reading frames (57). Open reading frames (ORFs) 1a and 1b encode two replicase polyproteins (pp1a and pp1ab) that are post-translationally processed by three ORF1a-encoded proteinases (nsp1, 2, and 4) to yield at least 13 nonstructural proteins (nsp1-12, including nsp 7α and 7β (55, 61, 69), and the remaining seven ORFs (2a, 2b and 3-7) encode structural proteins of the virus. These include four minor envelope proteins (E, GP2, GP3, and GP4 encoded by ORFs 2a, 2b, 3 and 4, respectively), two major envelope proteins (GP5 and M encoded by ORFs 5 and 6) and the highly conserved nucleocapsid protein (N encoded by ORF7; (56). The two major envelope proteins GP5 and M form a disulfide-linked heterodimer (15, 54) and the three minor envelope proteins GP2, GP3 and GP4 form a covalently associated heterotrimeric complex in the virion (63, 65). By independently knocking out the expression of each structural protein, it was shown that all major and minor structural proteins are required for the production of infectious progeny virus (46).

EAV is the causative agent of equine viral arteritis (EVA; (10, 19). Geographically and temporally different strains of EAV vary in the severity of the clinical disease they induce and in their abortigenic potential (1, 45, 49, 58). While most EAV infections are asymptomatic, some infected horses can exhibit clinical manifestations such as an influenza-like illness, abortion in pregnant mares, and pneumonia or pneumoenteritis in neonatal foals (18, 22, 23, 28, 43, 44, 58, 60). Following experimental infection of horses by the respiratory route, the virus initially replicates in both macrophages and endothelial cells in the
EA V is then transported to the draining lymph nodes, where it replicates and is released into the blood and lymphatic system for transportation throughout the body. The virus infects the smaller blood vessels, especially the arterioles, causing a panvasculitis (17, 35, 42). A number of studies have demonstrated that different strains of EA V vary significantly in their pathogenicity, with very different clinical outcomes upon experimental inoculation of horses (2, 5, 21, 43). The horse-adapted Bucyrus (VB) strain of EA V is highly velogenic and causes severe clinical disease with a case-fatality rate of 50-60% under experimental conditions (35, 51). Horses inoculated with virulent strains of EA V (e.g. VB and recombinant VB [rVBS] strains, and KY84) develop severe lymphopenia with a high-titered viremia (6x10^3-1x10^5 pfu/ml; (2, 5, 35, 44). In contrast, horses inoculated with the attenuated modified live virus (MLV) vaccine strain, or other avirulent strains of EA V (e.g. 030H and CA95G; (6, 49) develop a mild, transient lymphopenia with only a very low-titer viremia (≤1x10^1 pfu/ml; (20, 24, 37-40).

The modified live virus (MLV) vaccine (ARVAC®, Fort Dodge Animal Health) that is in current use in the United States and Canada (20, 37-40), was developed by serial passage of the VB strain of EA V in primary horse kidney cells (HK131 passages), primary rabbit kidney cells (RK111 passages) and equine dermis cells (ED24 passages) which resulted in attenuation of the virus (24). Previously, it has been shown that the VB strain was fully attenuated for horses by 116th passages in primary horse kidney cells (EA V HK116; (37, 38, 41). Subsequently, EA V HK116 virus was further passaged in HK cells for additional 15 times (HK131) and in two other cell lines (RK111 and ED24) to obtain the MLV vaccine virus (HK131, RK111, ED24) used in this study. There are 23 additional amino acid substitutions that accumulated in GP2, GP3, GP4, GP5 and M envelope proteins during cell culture passage of the HK116 virus to generate the current MLV strain (ARVAC®, Table 1) which may have contributed to further attenuation of the vaccine strain of EA V and increased the
safety of the vaccine for horses. Recently, using reverse genetics we showed that a chimeric
virus containing the nonstructural proteins of VB virus and the structural proteins of HK116
(rVBS/HK116 S; (66) strain has an attenuated phenotype in horses. The data showed that
critical amino acid substitutions in structural protein genes of HK116 virus were responsible
for attenuation of the VB strain.

To enhance fundamental understanding of the pathobiology of EAV infections it is
important to identify those peripheral blood mononuclear cells (PBMCs) that are most closely
associated with the virus during vascular transport and how genetic changes associated with an
attenuated phenotype alter the dynamics of virus/host-cell relationships in blood. Thus, we
hypothesized that VB and MLV strains differ in their ability to infect PBMCs and that the altered
tropism of the attenuated MLV strain of EAV in PBMCs is associated with amino acid changes
in the viral proteins. To test this hypothesis we used three previously described recombinant
viruses (rVBS (5), rVBS/HK116 S (66) and rMLV (67), as well as four newly generated
chimeric viruses (rVBS/MLV S, rMLV/VBS S, rMLV/VBS 234 and rMLV/VBS 56) and
infected ex vivo preparations of PBMCs collected from horses. The data suggested that the
difference in cellular tropism and virulence phenotype of VB and MLV strains is associated with
the collective interactions of both major (GP5 and M) and minor (GP2, GP3 and GP4) envelope
proteins of EAV. Furthermore, this study also demonstrated that CD3+ T lymphocyte tropism is
primarily determined by amino acid substitutions in the GP2, GP4, GP5 and M envelope
proteins but not the GP3 minor envelope protein. However, the macrophage tropism is mainly
determined by intricate interactions among GP2, GP3, GP4, GP5 and M envelope proteins of the
virus.
Materials and Methods

Cell lines. Equine pulmonary artery endothelial cells (EECs) were maintained in Dulbecco’s modified essential medium (Mediatech, Herndon, VA) with sodium pyruvate, 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT), 100U/ml of penicillin/streptomycin and 200mM L-glutamine (2, 5, 35). The rabbit kidney cells (RK-13, ATCC CCL-37; American Type Culture Collection, Manassas, VA) were grown in Eagle’s minimum essential medium with 10% ferritin supplemented bovine calf serum (Hyclone Laboratories, Inc., Logan, UT) and 100U/ml of penicillin/streptomycin (Gibco, Carlsbad, CA).

Horses. A total of 10 horses of different breeds (Thoroughbreds, n=4; Standardbreds, n=2; mixed breeds, n=4) were used in this study. The horses were maintained on pasture at the Department of Veterinary Science’s Maine Chance Farm, Lexington, KY. All horses were confirmed seronegative for EA V neutralizing antibodies using previously described protocols (53). The horses were clinically evaluated prior to collection of blood samples for isolation of peripheral blood mononuclear cells. Furthermore, complete blood counts and differential counts were performed to establish that all animals had normal blood cell values. Blood samples were collected according to a protocol approved by Institutional Animal Care and Use Committee at University of Kentucky, Lexington, KY.

Antibodies. To determine the phenotype of EA V infected mononuclear cells, the following monoclonal antibodies (MAbs) directed against different cell-type-specific surface molecules were used in this study: anti-equine CD3+ T lymphocytes (UC F6G; kindly provided by Dr. Jeff Stott, University of California at Davis; (7), anti-equine CD4+ T lymphocytes and anti-equine CD8+ T lymphocytes (CVS4 and CVS8, respectively; Serotec, Raleigh, NC; (33, 34). A MAb specific for human CD14 (Alexis Biochemicals, Lausen, Switzerland) that cross-reacts with equine CD14 (27) was used to identify equine monocytes and R-phycoerythrin.
(R-PE) conjugated anti-human CD21 (B-ly4; BD Pharmingen, San Jose, CA) previously shown to cross-react with equine B cells was used (36). R-phycoerythrin (R-PE)-conjugated F(ab’)2 fragment of goat anti-mouse IgG (Southern Biotech, Birmingham, AL) was used as a secondary antibody.

To detect EAV antigens in infected cells, MAbs against EAV nonstructural protein-1 (nsp1; MAb 12A4) and nucleocapsid protein (N; Mab 3E2) were used (35, 62). Mouse ascitic fluid containing MAbs 12A4 and 3E2 were purified using a Melon™ Gel IgG Spin Purification Kit (Pierce, Rockford, IL). Purified IgG was directly conjugated to Alexa Fluor® 488 (Invitrogen, Carlsbad, CA) following manufacturer’s instructions. Briefly, purified IgG from MAbs 12A4 and 3E2 were diluted with PBS to a concentration of 2 mg/ml and 1 M sodium bicarbonate (pH 8.3) was added. Subsequently, diluted IgG solution was incubated with Alexa Fluor® 488 reactive dye for 1 h at room temperature in the dark with slow stirring. The reaction mixture was loaded onto a fine size exclusion purification resin column for separation of labeled antibodies from unincorporated dye. Fractions of Alexa Fluor® 488 labeled antibodies were collected and stored at 4°C. To detect nonspecific binding, mouse IgG1 (MOPC; Sigma, St. Louis, MO) and Alexa Fluor® 488 conjugated mouse IgG1 isotype controls (Invitrogen, Carlsbad, CA) were used.

Viruses. Two strains of EAV, the virulent Bucyrus (VB) strain (ATCC VR-796; (10, 19) and the modified live virus (MLV) vaccine strain (ARVAC®, Fort Dodge Laboratories, Fort Dodge, IA, (24, 59), were used for in vitro study of identifying the EAV target cell population in PBMCs. Each virus was propagated once in EECs to prepare high titered working stocks as previously described (26, 47). Briefly, EECs infected with each virus were frozen at -80°C when 90-100% cytopathic effect (CPE) was observed. Cell lysates were clarified by centrifugation (500 ×g) at 4°C for 15 min, followed by ultracentrifugation (Beckman Coulter, Miami, FL) at 121,600 ×g through a 20% sucrose cushion in NET buffer (150 mM NaCl, 5
mM EDTA, and 50 mM Tris-HCl, pH 7.5) at 4°C for 4 h to pellet the virus. Purified preparations of each strain of EAV were resuspended in phosphate buffered saline (PBS; pH 7.4) and frozen at -80°C. Virus stocks were titrated by standard plaque assay in RK-13 cells and titers were expressed as PFU/ml (41).

Construction of chimeric cDNA clones of EAV and recovery of recombinant viruses.

Infectious cDNA clones of the virulent VB strain of EAV and modified live virus (MLV) vaccine strain of EAV (pEAVrVBS and pEAVrMLV, respectively) were recently constructed in our laboratory (5, 67). The *in vitro* and *in vivo* characterization of these viruses showed that they are phenotypically indistinguishable to that of their respective wild-type parental viruses. Five chimeric infectious cDNA clones, pEAVrVBS/HK116 S, pEAVrVBS/MLV S, pEAVrMLV/VBS S, pEAVrMLV/VBS 234 and pEAVrMLV/VBS 56 were constructed by using standard molecular biological techniques (5, 66, 68). The recombinant chimeric infectious cDNA clone pEAVrVBS/HK116 S was generated by substituting the genes (ORFs 2a to 7) encoding the structural proteins of pEAVrVBS by the corresponding genes from the HK116 virus (66). The recombinant chimeric infectious cDNA clone pEAVrVBS/MLV S was constructed by replacing the structural protein genes (ORFs 2a to 7) of the infectious cDNA clone pEAVrVBS (5), which was derived from the VB strain of EAV, with the corresponding genes of pEAVrMLV clone (67). A similar approach was used to construct the recombinant infectious cDNA clones pEAVrMLV/VBS S where the structural protein genes (ORFs 2a to 7) of the infectious cDNA clone pEAVrMLV were substituted with the corresponding genes of the pEAVrVBS clone; the pEAVrMLV/VBS 234 clone in which the minor envelope protein genes (ORFs 2a to 4) of the rMLV were replaced with the corresponding genes of pEAVrVBS; and the rMLV/VBS 56 clone in which the major envelope protein genes (ORFs 5 and 6) of the pEAVrMLV were replaced with the corresponding genes of pEAVrVBS.
The recombinant viruses, rVBS, rMLV, rVBS/HK116 S, rVBS/MLV S, rMLV/VBS S, rMLV/VBS 234 and rMLV/VBS 56, were generated by in vitro transcription of infectious viral RNAs from XhoI-linearized full-length infectious cDNA clones and electroporated into EECs following previously described protocols (5). Virus particles were harvested from cell culture supernatant at 48 to 72 h after electroporation when cytopathic effect (CPE) was evident, clarified of cell debris by centrifugation, and stored at -80°C in single-use aliquots (passage 0 [P0]). Recombinant viruses (P0) harvested from transfected EECs were used to prepare high titered working stocks by passing one more time in EECs (P1) as described above and purified viruses were used for in vitro infection studies.

Comparative amino acid sequence analysis and membrane topology prediction of EAV envelope proteins. The published sequences of rVBS, rMLV, HK116 were downloaded from the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html; accession numbers DQ846751 (5), EU586275 (67), and EU586274 (66), respectively). ORFs 2a, 2b, 3, 4, 5 and 6 were translated into amino acid sequences and aligned with the Vector NTI Advance™ 11 software (Invitrogen, Carlsbad, CA). Prediction of membrane topology for viral envelope proteins was performed using four most commonly used topology prediction methods available on the Internet: PSIPRED (http://www.psipred.net/psiform.html) (29, 30), TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) (31), TMPred (http://www.ch.embnet.org/software/TMPRED_form.html), TOPPRED (http://www.bioweb.pasteur.fr/seqanal/interfaces/toppred.html) (12). All user-adjustable parameters were left at their default values.

Preparation of peripheral blood mononuclear cell (PBMC) cultures. Blood (150-200ml) was collected aseptically from each of 10 horses using vacutainer tubes containing 15% EDTA solution (Kendall Healthcare, Mansfield, MA). Peripheral blood mononuclear cells (PBMC) were isolated from the buffy coat fraction by centrifugation through Ficoll-
Paque Plus™ (Amersham Biosciences, Piscataway, NJ) at 500 × g for 30 min at 25°C. The PBMC layer was collected, washed twice with PBS (pH 7.4) by centrifuging at 100 × g for 10 min to eliminate the platelets. The cells were resuspended in complete RPMI (c-RPMI) 1640 medium (Gibco, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT), 2 mM glutamine, 100U/ml penicillin/streptomycin and 55 µM 2-mercaptoethanol and counted using a Visell Counter-XR (Beckman Coulter, Miami, FL). The adherent and non-adherent cell populations were separated by plating out the PBMCs in 100 mm tissue culture dishes (Corning, Corning, NY). Briefly, the cells were incubated at 37°C for 4 h to allow adherent cells to attach, and after incubation, the unattached cells were removed, centrifuged and resuspended to be counted and plated out as a non-adherent cell population in 100 mm tissue culture dishes containing 5ml of c-RPMI medium. The adherent cells were washed twice with PBS to remove remaining non-adherent cells and replaced with 5 ml of fresh c-RPMI medium. Both adherent and non-adherent cells were incubated at 37°C until infected with EAV.

**Sorting of lymphocytes and monocytes.** Positive selection of five different cell populations (CD3⁺, CD4⁺, CD8⁺, CD21⁺ and CD14⁺ cells) was performed using aseptically obtained horse blood and a MoFlo Cell Sorter (Dako Cytomation, Glostrup, Denmark). Briefly, 5 × 10⁷ PBMCs were incubated with specific MAbs against equine CD3, CD4 or CD8 for T lymphocytes and human CD14 that cross-react with equine monocytes. Equine B lymphocytes were sorted by two-way sorting using R-PE conjugated anti-human CD21 antibody that cross react with equine B lymphocytes on cells already incubated with anti-equine CD8 MAb. All antibodies were incubated on ice in sorting buffer (1% FBS in Hanks' Balanced Salt Solution, pH 7.4; Gibco, Carlsbad, CA) for 20 min. After incubation with primary antibody, cells were washed and stained with goat anti-mouse IgG₁-R-PE (Southern Biotechnology Inc., Birmingham, AL) or goat anti-mouse IgG-FITC (Caltag...
Laboratories, Burlingame, CA) on ice for 20 min.

**In vitro infection of lymphocytes and monocytes.** Cultures of lymphocytes and monocytes were infected with either wild-type (VBS and MLV) or recombinant viruses (rVBS, rMLV, rVBS/HK116 S, rVBS/MLV S, rMLV/VBS S, rMLV/VBS 234 and rMLV/VBS 56) of EAV at an m.o.i. of 2. As negative controls, mock infected lymphocytes and monocytes were cultured under identical conditions.

**Dual-color immunofluorescence staining and flow cytometry.** For two color immunofluorescence staining of wild-type or recombinant virus infected cells, lymphocytes (1×10^6 cells) were incubated on ice for 30 min with MAb specific for equine CD3^+^, CD4^+^ or CD8^+^ T cells and R-PE conjugated anti-human CD21 antibody for B cells. Blood-derived monocytes (1×10^6 cells) were stained with anti-human CD14 monoclonal antibody. After washing, cells were incubated with secondary R-PE-conjugated goat anti-mouse IgG1 (Southern Biotech, Birmingham, AL) for 30 min on ice. After washing to remove unbound secondary antibody, cells were fixed with 4% paraformaldehyde and then washed once in PBS-saponin buffer (PBS [pH 7.4] supplemented with 1% FBS, 0.1% saponin and 0.1% sodium azide). Intracellular staining for EAV antigen was performed using an Alexa Fluor® 488 conjugated anti-EAV nsp1 MAb 12A4 or anti-EAV N MAb 3E2 in PBS-saponin buffer and incubated on ice for 30 min. After incubation, washed cells were resuspended in PBS containing 0.5% paraformaldehyde for two-color cytometric acquisition using a FACSCalibur (Becton Dickinson, San Jose, CA). Lymphocytes and monocytes were gated and selected based on forward and side-scatter parameters of analysis. Cells were evaluated by a two-color plot of anti-EAV antigen (FL-1) vs. cell surface antigen (FL-2), and the percentage of CD3^+^, CD4^+^, CD8^+^, CD21^+^ or CD14^+^ and EAV antigen positive cells was determined by CellQuest™ quadrant statistics. Results were expressed as the percentage of lymphocytes or monocytes infected with EAV, after subtraction of the non-specific staining of mock-
infected cells.

**Virus growth curve and quantitative EAV real-time TaqMan® RT-PCR assay.** Sorted CD3⁺, CD4⁺, CD8⁺ T lymphocytes, CD21⁺ B lymphocytes and CD14⁺ monocytes were infected with VB or MLV strains of EAV at an m.o.i. of 2. Tissue culture supernatants were collected at 0, 1, 6, 12, 18, 24, 36, 48, 60 and 72 hpi for one-step growth curve and quantitative EAV real-time TaqMan® RT-PCR assays. For one-step growth curve analysis, virus titers in tissue culture supernatants were determined according to the method of Reed and Muench (52), and expressed as 50% tissue culture infective dose (TCID₅₀)/50 µl. To detect EAV nucleic acids, a one-tube real-time TaqMan® RT-PCR assay was performed using the TaqMan® One-Step RT-PCR Master Mix (Applied Biosystems, Foster City, CA) in an Applied Biosystems 7500 Fast Real-Time PCR System according to a previously published protocol (3, 32). The copy numbers of EAV molecules were determined by absolute quantification with an IVT ORF7 RNA standard curve as described previously (3, 32).

**Statistical analysis.** The student’s t-test was used to establish significant difference among infected lymphocyte subpopulations and monocytes between group A and B horses. Statistical analysis was performed using Sigma Plot 11 (Systat Inc., Richmond, CA).

**Nucleotide sequence accession numbers.** The nucleotide sequences of the rVBS/HK116 S, rVBS/MLV S, rMLV/VBS S, rMLV/VBS 56 and rMLV/VBS 234 were deposited in GenBank under the accession numbers GU732202, GU732201, GU732200, GU732199 and GU732198, respectively.

**Results**

**Differences in susceptibility of equine peripheral blood mononuclear cells (PBMCs) to infection with virulent and attenuated strains of EAV**

To investigate the susceptibility of equine PBMCs to virulent and attenuated strains
of EAV, PBMCs collected from ten randomly selected horses were infected either with the VB or MLV strains of the virus. Dual fluorescent antibody staining of PBMC cultures was performed using a panel of leukocyte differentiation antigen-specific monoclonal antibodies (MAbs) specific for pan CD3+ T lymphocytes, CD4+ helper T lymphocytes, CD8+ cytotoxic T lymphocytes and CD21+ B lymphocytes, as well as a MAb specific for the EAV nsp1. Dual immunofluorescence labeling with CD3 specific MAb and antibody against EAV nsp1 indicated that CD3+ T lymphocytes recovered from six of the ten tested horses could be infected with the VB strain of EAV (Fig. 1A). At 12 hpi, the mean percentage of CD3+ T lymphocytes double-labeled with cell surface antibody and MAb to EAV nsp1 antigen was 0.5% (± 0.02). The mean percentage of double-labeled CD3+ T lymphocytes increased to 5.5% (± 1.0) by 24 hpi. The EAV nsp1 antigen expression peaked with a mean percentage of 6.6% (± 0.8) of double-labeled CD3+ T cells at 36 hpi and decreased to 4% (± 0.4) at 48 hpi. Interestingly, none of the CD3+ T lymphocytes from the remaining four horses were infected with the VB strain of EAV (Fig. 1B). Based on the phenotype of CD3+ T lymphocyte subpopulations to in vitro infection with VB strain of EAV, the ten horses could be divided into susceptible (Group A; 6/10) and resistant (Group B; 4/10) groups. Contrary to infection with the VB strain, T lymphocytes from none of the 10 donor horses were susceptible to infection with the MLV vaccine strain of EAV (Fig. 1A and B).

In an attempt to define the T lymphocyte subpopulation susceptible to VB infection, lymphocytes from Group A horses were infected with the VB strain and were stained with either CD4 or CD8 specific MAb and antibody to EAV nsp1. Similar to CD3+ T lymphocytes, the mean percentage of double-labeled CD4+ T cells increased to 4.4% (± 0.8) by 24 hpi. The EAV nsp1 antigen expression peaked at 36 h after inoculation with a mean percentage of 5% (± 0.7) of double-labeled CD4+ T cells which decreased subsequently to 2.8% (± 0.3) at 48 hpi (Fig. 2A). The mean percentage of double-labeled CD8+ T cells...
ranged from 0.03 to 1.1% ([± 0.03 to 0.2]; Fig. 2B) through to the completion of the experiment. These findings indicated that the majority of CD3+ T lymphocytes infected with the VB strain were CD4+ T lymphocytes rather than CD8+ T lymphocytes. Not surprisingly, in a similar experiment neither CD4+ nor CD8+ T lymphocytes from the four horses in group B could be infected with the VB strain of EAV (data not shown). EAV nsp1 antigen expression was not detected in the CD21+ B lymphocyte cultures from either group A or B horses inoculated with either the VB or MLV vaccine strains (Fig. 2C) indicating that B lymphocytes are not susceptible to EAV infection. In summary, viral antigen could be detected primarily in CD4+ T lymphocyte subpopulations from Group A horses infected with VB strain of EAV. These data showed that only CD4+ T lymphocytes from some horses are susceptible to infection with the VB strain but none of them are susceptible to infection with MLV strain indicating that attenuation of the VB strain has altered its cellular tropism.

To investigate whether monocytes are equally susceptible to infection with VB and MLV strains of EAV, dual immunofluorescence staining was performed using MAbs specific for CD14+ monocytes and MAb specific for the EAV nsp1. Using the adherent cells obtained from PBMCs derived from two of each group of horses, the percentage of CD14+ monocytes ranged from 60 to 75% depending on the preparation. Double-labeled flow cytometric analysis showed that monocytes from all of the tested horses could be infected with VB and MLV strains of EAV (Figure 3). However, the mean percentage of MLV infected cells was significantly lower and remained near the lower limit of detection throughout the experimental time course compared to those detected in VB infected monocytes (Figures 3A and B) from both group of horses. In the case of cultures infected with the VB strain, the maximum mean percentage of cells expressing EAV nsp1 antigen in monocytes from Group A horses (n=2) was approximately 31.8% (± 7.2) at 12 hpi, after which it had declined sharply by (1.8 ± 0.2%) 48 hpi (Figure 3A; Group A). Similar
patterns were noted for monocytes purified from the Group B horses (n=2; Figure 3B; Group B). However, the overall mean percentage of cells expressing EAV antigen was significantly lower in monocytes from the Group B horses (14.3% ± 7.3 at 12 hpi and 11.3% ± 4.7 at 24 hpi) compared to monocytes from the susceptible horses (Group A) at 12 and 24 hpi (Fig. 3A and B). The mean percentage of infected monocytes in MLV inoculated cultures was minimal at 12 hpi after which it gradually increased. Although similar patterns of infection were shown for cells derived from horses in groups A and B, the mean percentage of infected cells in the case of the former was significantly higher than in the case of the latter (Fig. 3A and B). Furthermore, these findings confirm that not only T lymphocytes but also monocytes differ in their susceptibility to infection with VB and MLV strains of EAV.

Expression of the structural proteins of EAV in PBMCs infected with virulent and attenuated strains of EAV

One of the key findings of this study is the susceptibility of CD3+ T (predominantly CD4+ T) lymphocytes of Group A horses to VB infection as determined by the expression of the nsp1 protein. To investigate whether viral structural protein genes are also expressed in infected cultures, two-color flow cytometric analysis was performed using MAbs specific for cell surface markers and MAb specific for the EAV nucleocapsid (N) protein. When T lymphocytes isolated from horses in Group A were infected with the VB strain, both nsp1 and N protein expression were detected in CD3+, CD4+ and CD8+ T lymphocytes (Fig. 4, panels a through c [nsp1] and panels d through f [N]). In contrast, CD3+, CD4+ and CD8+ T lymphocytes were completely refractory to infection with the MLV strain since neither nsp1 nor N protein expression could be observed in inoculated cultures (Fig. 4, panels g through i [nsp1] and panels j through l [N]). The data indicated that a virus replication cycle, at least up to expression of structural proteins, occurred in VB infected T lymphocytes but not in
MLV infected T lymphocytes of Group A horses. However, release of progeny virus particles could not be detected in VB inoculated cultures of sorted CD3⁺, CD4⁺ and CD8⁺ T lymphocytes from any of tested horses based on virus one step growth curve and quantitative real-time RT-PCR (qRT-PCR) results (data not shown).

Furthermore, we also investigated the expression of N protein in monocytes infected with the VB and MLV strains of EAV (Fig. 5A). In contrast to T lymphocytes, expression of both nsp1 and N protein were detected in monocytes infected with VB and MLV strains. Not surprisingly, the percentage of monocytes expressing nsp1 and N proteins was significantly greater in VB infected cultures (24.8% ± 4.4 and 18.3% ± 1.8, respectively [panels a and b]) compared to the corresponding percentage of cultures infected with the MLV strain (5.5% ± 1.9 and 3.7% ± 1.8, respectively; Fig. 5A [panels c and d]). Additionally, viral titers and viral nucleic acid copy numbers from sorted CD14⁺ monocytes inoculated with VB and MLV strains of EAV confirmed the findings from double-staining flow cytometric analysis (Fig. 5B and C, respectively). In monocyte cultures inoculated with the VB strain, viral titers increased gradually by 18 hpi reaching a maximum titer of approximately 10⁵ TCID₅₀/50 µl at 36 hpi. After reaching this peak level, titers gradually declined to 10⁴ TCID₅₀/50 µl by the final sample collection time at 72 hpi. Although, productive viral replication also occurred in MLV inoculated monocytes, viral titers were lower than those detected in VB inoculated cells (Fig. 5B). Interestingly, while MLV titers remained low until 48 hpi, they showed a significant increase by 72 hpi. The trends in the viral growth curves were similar to those observed in nucleic acid copy numbers quantified by qRT-PCR (Fig. 5C). Monocytes infected with the VB strain had increased viral nucleic acid copy numbers from 18 hpi reaching a maximum after 48 hpi in both groups of horses. In contrast to VB infected monocytes, viral nucleic acid copy numbers in monocytes infected with the MLV strain remained low throughout the time course of the experiment, though they
had started to increase at 72 hpi which was consistent with the viral growth curve results (Fig. 5C). These data confirmed that the MLV strain replicates slower and to lower titers in monocytes compared to the VB strain of EAV in Group A horses.

**Infection of CD3^+ T lymphocytes and CD14^+ monocytes with EAV recombinant chimeric viruses**

A marked disparity in the ability of VB and MLV strains to infect subpopulations of susceptible CD3^+ T lymphocytes and monocytes (Group A horses) suggested that there is a clear difference in cellular tropism between the two strains. In our preliminary studies, the recombinant viruses, rVBS and rMLV, had similar growth kinetics in equine endothelial cells (EECs; data not shown) and an equivalent capacity for lymphocyte infectivity as confirmed by flow cytometry analysis (Fig. 6; panels a through d [rVBS] and i through l [rMLV]) when compared to their respective wild-type parental viruses, VB and MLV. To identify the viral proteins responsible for the differential cellular tropism between VB and MLV, we attempted to assess the impact of amino acid changes accumulated during cell culture passage of HK116 strain, which is fully attenuated for horses as compared to the VB strain. Therefore, we used the previously described rVBS/HK116 S chimeric virus (66) containing the structural proteins of EAV HK116 virus in backbone of rVBS genome to infect PBMCs from Group A horses. When susceptible T lymphocytes were inoculated with chimeric rVBS/HK116 S, the number of T lymphocytes expressing EAV nspl antigen decreased significantly compared to rVBS (2.3% to 0.2%; Fig. 6; panels e through g). In contrast, the percentage of infected monocytes was comparable to that using the rVBS virus indicating that the tropism of the HK116 strain had changed for lymphocytes but not for monocytes following 116 passages in primary horse kidney cells (Fig. 6; panel h). In summary, these data showed that amino acid substitutions in the structural protein genes of HK116 strain changed CD3^+ T lymphocyte tropism of the virus without any significant effect on its CD14^+...
monocyte tropism.

In an attempt to further identify the specific viral proteins responsible for the differential tropism among VB, HK116 and MLV strains in equine PBMCs, we generated a new panel of four recombinant chimeric viruses using the infectious cDNA clones of the VB and MLV strains (rVBS and rMLV, respectively). The panel consisted of reciprocal chimeric viruses containing interchanged structural and nonstructural protein genes of the VB and MLV strains (Fig. 6). When susceptible lymphocytes and monocytes were infected with rVBS/MLV S and rMLV/VBS S viruses, rVBS/MLV S did not infect any CD3+ T lymphocytes and only replicated in CD14+ monocytes at a very low level which was identical to that observed in rMLV infection (Fig. 6; panels m through p). In contrast, the rMLV/VBS S infected and replicated in both CD3+ T lymphocytes and CD14+ monocytes similar to rVBS but there was a reduction in viral protein expression in lymphocytes compared with that observed in cells infected with rVBS virus (Fig. 6; panels q through t). These results suggest that the structural proteins of the VB strain are responsible for determining its tropism for lymphocytes and monocytes. Furthermore, comparison of dual-color flow cytometric data of PBMCs infected with rVBS/HK116 S and rVBS/MLV S showed significant differences in CD14+ monocyte infectivity, indicating that amino acid substitutions that occurred during further cell culture passage of HK116 may have contributed to the change in monocyte tropism. Taken together, the data suggest that viral tropism for CD3+ T lymphocyte and CD14+ monocyte was altered by amino acid changes in structural proteins of EAV.

To further investigate the role of minor and major envelope proteins of EAV in cellular tropism, additional recombinant viruses, rMLV/VBS 234 and rMLV/VBS 56, were generated with the MLV infectious cDNA clone as the viral backbone and used for in vitro infection of susceptible lymphocytes and monocytes. Recombinant rMLV/VBS 234 virus
contained the genome sequence identical to that of the rMLV virus except for ORFs 2a, 2b, 3 and 4 (encoding for E, GP2, GP3 and GP4 minor envelope proteins) that were replaced by the corresponding regions of the rVBS virus. In the case of rMLV/VBS 56 virus, whose ORFs 5 and 6 (encoding for GP5 and M major envelope proteins) were replaced with the corresponding genes of rVBS in the rMLV backbone (Fig. 6). In contrast to expectations, neither rMLV/VBS 234 nor rMLV/VBS 56 chimeras were able to infect T lymphocytes (Fig. 6; panels u through w [rMLV/VBS 234] and panels y through z’ [rMLV/VBS 56]). However, comparisons in monocytes showed that while the percentage of cells infected with rMLV/VBS 56 virus was similar to that of rMLV and rVBS/MLV S viruses, infection rates for rMLV/VBS 234 virus were significantly lower (Fig. 6; panels z” and x, respectively). Therefore, the higher relative fluorescence intensity values observed in monocytes with rMLV/VBS 56 (Figure 6; panel z”) compared to rMLV/VBS 234 (Fig. 6; panel x) suggest GP5 and M amino acid sequences may play a more critical role than those of E, GP2, GP3 and GP4 in facilitating monocyte infections. Taken together, these data demonstrate the minor envelope glycoproteins as well as the two major envelope proteins play a critical role in determining monocyte tropism.

**Comparative amino acid sequence analysis of GP2, GP3, GP4, GP5 and M proteins of rVBS, HK116 and rMLV viruses**

The reverse genetic studies using recombinant chimeric viruses clearly indicated that collective interactions among all EAV envelope proteins are required for efficient infection of susceptible subpopulations of CD3+ T lymphocytes and CD14+ monocytes, and these interactions were disabled or altered by the amino acid changes which had occurred during extensive serial passage of the VB strain resulting in the HK116 and MLV vaccine strains of EAV. Accordingly, the nucleotide sequences of ORFs 2 to 6 from VB, HK116 and MLV strains have been translated, aligned and analyzed in the context of the predicted topography.
of each viral structural protein. The E minor envelope protein encoded by ORF2a was conserved (100% identity) among VB, HK116 and MLV strains and therefore it is almost certainly not responsible for the differences in tropism among the three EAV strains (Table 1). When compared to the VB strain, the GP2, GP4, GP5 and M envelope proteins of HK116 strain had several amino acid substitutions (2, 1, 3 and 3 substitutions respectively; Table 1). With the exception of amino acid substitutions in the M protein and one amino acid substitution in the GP2 protein (223R→P) of HK116 virus, all the amino acid changes were located in the ectodomain of these proteins (Table 1 and Fig. 7). Interestingly, in contrast to GP2, GP4, GP5 and M envelope proteins, the amino acids of the GP3 protein of HK116 strain were identical to the parental VB strain. The data indicated that loss of CD3⁺ T lymphocyte tropism of HK116 virus is primarily due to substitutions involving the GP2, GP4, GP5 and M envelope proteins rather than the GP3 minor envelope protein. However, further cell culture passage of this HK116 strain resulted in numerous other non-synonymous amino acid substitutions in ORFs 2b, 3-6 (Table 1, Fig. 7). All of these substitutions appear to be non-conservative and therefore are likely to affect secondary and tertiary structure which may change the interactions between these envelope proteins (Fig. 7). Analysis of ORF3 demonstrated a U→C substitution at position 10,795 in rMLV resulting in the removal of the normal stop codon present in rVBS and HK116 and permitting the addition of five amino acids to the carboxyl-terminus of GP3. Furthermore, there are three other non-synonymous nucleotide substitutions between rMLV and rVBS (as well as the intermediate HK116 strain) in ORF3 of which leucine to serine at amino acid position 123 and cysteine to tyrosine at position 160 appear to be non-conservative (Table 1, Fig. 7). In common with ORF3, all four non-synonymous nucleotide substitutions in ORF4 are predicted to occur in the ectodomain of GP4, a trend also observed in ORF5 where six of the eight non-synonymous substitutions occur within the same predicted domain for GP5 (Table 1, Fig. 7).
Therefore, it appears as if most of the variation in amino acid content between the structural proteins of rVBS and rMLV occurs in those regions that could interact directly with host-cell receptor molecules. An exception to this is in ORF6 where all seven non-synonymous nucleotide substitutions between rMLV and rVBS are predicted to occur in the endodomain or transmembrane-spanning domain of the M protein. During extensive cell culture passage, the second putative N-linked glycosylation site at amino acid position 81 (81N>D) in GP5 was lost leaving the GP5 of MLV with only the conserved putative N-linked glycosylation site at amino acid position 56. Taken together, these data clearly demonstrate that the failure of MLV to infect CD3⁺ T lymphocytes was determined by the amino acid changes in the GP2, GP4, GP5 and M proteins but not the GP3 protein. In contrast, the reduced monocyte tropism of the MLV strain appears to be due to the amino acid changes in the GP3 protein. However, additional amino acid substitutions in GP2, GP4, GP5 and M protein may also have contributed to the altered monocyte tropism. Since the nucleocapsid protein (N) encoded by ORF7 is not exposed on the virus surface, it is unlikely that this protein has contributed to the change in cellular tropism of EAV. Therefore, it is more likely that tropism of CD3⁺ T lymphocytes and CD14⁺ monocytes was altered due to the changes in major and minor envelope proteins of EAV.

**Discussion**

In contrast to attenuated strains of EAV, highly pathogenic strains are highly cell-associated with PBMCs and cause high titered viremia (2, 5, 35). However, until now the interaction between EAV and PBMCs has not been fully characterized and the specific cell types infected with EAV were not identified. Therefore, the primary objective of this study was to unequivocally establish which components of the PBMC population were susceptible to EAV infection and then identify the viral proteins responsible for infection of these cells.
Based on dual-color flow cytometric analysis and conventional viral titration assay along with qRT-PCR on infected cell culture fluids, we demonstrated that the predominant host cell type susceptible to virulent VB strain of EAV are CD14\(^+\) cells of the monocyte/macrophage lineage. Furthermore, under ex-vivo culture conditions these cells were permissive for complete productive replication of the VB strain. Although this is an important finding, it is perhaps not surprising in view of the fact EAV antigens have been found in alveolar macrophages in infected horses (4, 48) and that all other members of the Arteriviridae family are monocyte/macrophage tropic (50). However, the finding that the VB strain can infect CD3\(^+\) T lymphocytes (predominantly CD4\(^+\) T cells) of some but not all horses is the first report of a member of this virus family being associated with T lymphocytes. Surprisingly, there was a clear difference in the horse population based on the susceptibility of their CD3\(^+\) T lymphocytes and CD14\(^+\) monocytes with in vitro VB infection. As a result, horses were categorized as Group A in which almost one third of monocytes along with CD3\(^+\) T lymphocyte (mainly CD4\(^+\) T cells) subpopulations were susceptible to infection with the virulent EAV strain or as Group B in which monocyte susceptibility was only half of that observed in Group A horses and all CD3\(^+\) T lymphocyte subpopulations were resistant to EAV infection. Based on this finding, we assumed that horse’s genetic background may play a significant role in determining the clinical outcome of primary infection with EAV as reported in other species (8, 9). Efforts are underway in our laboratory to investigate if there is a genetic basis for the differences between horses in the susceptibility of their monocytes and CD3\(^+\) T lymphocytes to VB infection by analyzing possible associations with single nucleotide polymorphisms (SNP). Furthermore, an in vivo study is planned in which Group A and B horses are infected with rVBS to establish the relationship between PBMCs susceptibility and the severity of clinical illness or EVA, respectively.

Expression of nsp1 and N proteins in CD3\(^+\) T lymphocytes demonstrated the VB
strain undergoes initial replication steps such as binding/entry, uncoating and translation of nonstructural and structural proteins. However, production of progeny virus was not evident in sorted CD3⁺, CD4⁺ and CD8⁺ T lymphocytes from any of the tested horses based on the virus one-step growth curve results or qRT-PCR results despite synthesis of the full complement of viral proteins. This indicates that the number of progeny virions generated is either below the limit of detection, a possible reflection of the relatively small numbers of T lymphocytes infected (about 5%), or there is a blockage at a later stage in viral assembly and/or release. It remains to be determined if these cells represent “dead-end” hosts or if they are fully permissive. In contrast to the VB strain, expression of MLV nspl or N could not be detected in CD3⁺ T lymphocytes from any tested horses demonstrating that the amino acid substitutions that occurred during the attenuation process of the VB strain prevented entry and replication of MLV strain in these cells. In addition, the number of CD14⁺ monocytes expressing viral nspl or N proteins following infection with the attenuated MLV strain was significantly less than observed with VB virus.

Data derived from this study suggested that the amino acid substitutions in major and minor envelope proteins that occurred during cell culture passage of the VB strain contributed to the altered tropism of MLV virus for CD3⁺ T lymphocytes and CD14⁺ monocytes. In an attempt to identify the viral proteins associated with infection of T lymphocytes and greater numbers of monocytes, we generated a panel of recombinant chimeric viruses in which the structural and nonstructural protein genes of the parental rVBS were replaced with corresponding genes of the rMLV strain. In vitro evaluation of rMLV/VBS chimeric virus demonstrated conclusively that cell tropism was determined by envelope proteins of the virus. The role of the viral envelope proteins in CD3⁺ T lymphocyte and macrophage tropism was further defined by using recombinant chimeric viruses in which sequences encoding the minor envelope glycoproteins (GP2, GP3 and GP4)
or major envelope proteins (GP5 and M) of the MLV strain were replaced with the corresponding genes of rVBS in an rMLV backbone (designated rMLV/VBS 234 and rMLV/VBS 56, respectively). Recombinant chimeric viruses derived from these infectious cDNA clones were unable to infect T lymphocytes and numbers of monocytes expressing nsp1 were either lower (rMLV/VBS 234) or equivalent (rMLV/VBS 56) to those observed when these cells were infected with parental rMLV. For the first time evidence is presented suggesting that infection of T lymphocytes along with the ability to infect relatively large numbers of monocytes is dependent on cooperative interactions between five out of the six envelope proteins of EAV (GP2, GP3, GP4, GP5 and M). This, in turn, suggests that the infection process of these cell types is complex, possibly involving multiple receptor and/or receptor accessory molecules. Furthermore, the data also indicated that during extensive sequential cell culture passage, viral envelope protein genes have coevolved resulting in a synergistic effect on cellular tropism. Interestingly, based on findings with the rVBS/HK116 S chimeric virus, the GP3 protein is not associated with altered tropism for CD3⁺ T lymphocytes. Substitutions in the other two minor envelope glycoproteins (GP2 and GP4) and the two major envelope proteins (GP5 and M), however, play a critical role in changing the tropism for these cells. The dual-color flow cytometry data clearly demonstrated that the HK116 strain was significantly different from both VB and MLV strains. By 116th passages of the VB strain in primary HK cells, the virus lost its permissiveness for CD3⁺ T lymphocytes but its ability to infect CD14⁺ monocytes remained similar to the parental VB strain. Further cell culture passage of HK116 virus in different cell lines (HK15, RK111 and ED24) to obtain the MLV strain, resulted in significant reduction in its ability to infect macrophages (27% to 5%; Fig. 6; panels h and l, respectively). However, it is important to note that the attenuated phenotype of EAV HK116 for horses correlates with the loss of permissiveness for T lymphocytes while the ability to infect
monocytes remains similar to that of the parental VB strain. This suggests that either directly or indirectly tropism for T lymphocytes has a significant role in the pathogenesis of VB infections \textit{in vivo}. Therefore, the susceptibility of CD3$^+$ T lymphocytes to virulent and avirulent field strains of EAV and their role in the pathogenesis of EVA warrants further in-depth investigation.

Interestingly, based on comparative amino acid sequence and predicted membrane topology of the envelope proteins, the majority of the amino acid variations between VB and MLV viruses, as well as HK116, are located within the predicted ectodomain of each envelope protein, except for the M protein, in which the changes are in the transmembrane and cytoplasmic domains (Fig. 7). It is known that GP5 and M exist in the virion as a heterodimer while the minor envelope proteins (GP2, GP3 and GP4) occur in particles as heterotrimeric complexes constituting a virion-exposed structure that is predicted to mediate binding of the virion to the primary receptor (65). Formation of the heterotrimeric minor envelope complex is dependent on intramolecular cysteine bonding with GP2 and GP3 being linked via interactions with GP4 (63). The existence of these complexes coupled with the fact that most of the amino acid substitutions appear to be non-conservative, suggest that the cell tropism of MLV and VB strains is determined by conformational differences in structural envelope proteins that are manifested at the tertiary and quaternary levels. Therefore, the amino acid substitutions located within the various ectodomains may have direct and indirect effects on interactions with host cells as well as associations between the viral structural proteins. Consistent with our findings, a recent published study on porcine reproductive and respiratory syndrome virus has shown that inter-glycoprotein interactions are critical for mediating interactions with the receptor responsible for virus entry into host cells (14). Specifically, it has been demonstrated strong interaction between GP4 and GP5, as well as weak interactions among other minor envelope proteins resulting in the formation of
multiprotein complex. Furthermore, in a previous study we have also demonstrated that change in cellular tropism and establishment of persistent infection in HeLa cells by VB strain of EAV was associated with amino acid substitutions in the envelope proteins (68). Reverse genetic studies further confirmed that substitutions in the minor envelope proteins E and GP2 or GP3 and GP4 alone were unable to change cellular tropism and establishment of persistent infection in HeLa cells but recombinant viruses with combined substitutions in the E, GP2, GP3 and GP4 as well as a single amino acid substitution in the GP5 were able to alter cellular tropism of VB strain of EAV and favor establishment of persistent infection in HeLa cells. Nevertheless, the situation is further complicated by the fact so little is known about the EAV receptor and whether this is restricted to a single molecule or if the virus can use multiple alternatives but potentially cell-type specific molecules.

In summary, for the first time we demonstrate that extensive cell culture passage of the VB strain of EAV to produce the MLV vaccine strain has altered its cellular tropism for equine PBMCs. Evaluation of chimeric viruses demonstrated that cellular tropism in PBMCs is determined by the amino acid sequence of viral envelope proteins. Specifically, the data suggest that the GP2, GP4, GP5 and M envelope proteins play a critical role in CD3+ T lymphocyte tropism while three minor envelope proteins (GP2, GP3 and GP4) as well as the GP5 and M major envelope proteins determine the CD14+ monocyte tropism of EAV.

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References


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Figure legends

Figure 1. Differences in susceptibility of CD3⁺ T lymphocytes to infection with VB and MLV strains of EAV. Dual color immunofluorescence flow cytometric analysis was performed at various hours post-infection (hpi). (A) Values represent the mean percentage of infected CD3⁺ T cells (± S.E.M) with VB (black circles) and MLV (white circles) for 6 horses with T lymphocytes susceptible to EAV VB infection (Group A). Percentages of infected cells at the indicated times (data points on the curve) were determined by using dot plots derived from dual fluorescence flow cytometric analyses of cells (representative dot plots of VB infection, insets a through d; MLV infection, insets e through h). (B) Values represent the mean percentage of infected CD3⁺ T cells (± S.E.M) with VB (black circles) and MLV (white circles) for 4 horses with T lymphocytes resistant to EAV VB infection (Group B) as determined by using dot plots derived from dual fluorescence flow cytometric analyses of cells (representative dot plots of VB infection, insets a through d; MLV infection, insets e through h).

Figure 2. Susceptibility differences of CD4⁺ and CD8⁺ T lymphocytes and CD21⁺ B lymphocytes to infection with VB and MLV strains of EAV. Dual color immunofluorescence flow cytometric analysis was performed at various time p.i. Values represent the mean percentage of (A) infected CD4⁺ T cells, (B) infected CD8⁺ T cells and (C) infected CD21⁺ B cells (± S.E.M) with VB (black circles) and MLV (white circles) strains of EAV. Percentages of infected cells at the indicated times (data points on the curve) were determined by using dot plots derived from dual fluorescence flow cytometric analyses of cells (representative dot plots of VB infection, insets a through d; MLV infection, insets e through h).

Figure 3. Infection of monocytes with VB and MLV strains of EAV. (A and B) Dual color immunofluorescence flow cytometric analysis was performed at various time p.i.
Values represent the mean percentage of infected monocytes (± S.E.M) with VB (black circles) and MLV (white circles) for (A) 2 horses with T lymphocytes susceptible to EAV VB infection (Group A) and (B) 2 horses with T lymphocytes resistant to EAV VB infection (Group B). Percentages of infected cells at the indicated times (data points on the curve) were determined by using dot plots derived from dual fluorescence flow cytometric analyses of cells (representative dot plots of VB infection, insets a through d; MLV infection, insets e through h).

**Figure 4. Detection of EAV nucleocapsid (N) protein expression in T lymphocytes infected with VB strain.** The CD3⁺, CD4⁺ and CD8⁺ T lymphocytes from Group A horses infected with VB and MLV strains of EAV were examined by dual-color immunofluorescence flow cytometric analysis using MAbs against EAV nsp1 (MAb 12A4) and N (MAb 3E2) proteins and MAbs for cell specific cell surface antigens at 24 hpi. (Panels a through c) Lymphocytes infected with VB strain were stained with anti-EAV nsp1 AF488 MAb and one of the specific antibodies for T lymphocytes including MAbs to CD3, CD4, and CD8, respectively. (Panels d through f) Dot plots derived from same cultures stained with anti-EAV N AF488 MAb and specific antibodies for CD3⁺, CD4⁺ and CD8⁺ T lymphocytes, respectively. (Panels g through i) Lymphocytes infected with MLV strain were stained with anti-EAV nsp1 MAb and specific antibodies for T lymphocytes including MAbs to CD3, CD4, and CD8, respectively. (Panels j through l) Dot plots derived from same cultures stained with anti-EAV N AF488 MAb and specific antibodies for CD3⁺, CD4⁺ and CD8⁺ T lymphocytes, respectively.

**Figure 5. Replication of VB and MLV strains of EAV in blood-derived monocytes of Group A horses.** (A) The CD14⁺ monocytes infected with VB and MLV strains of EAV were examined by dual-color immunofluorescence flow cytometric analysis using MAbs against EAV nsp1 (12A4) and N (3E2) proteins and MAbs for cell specific cell surface antigens at 24
hpi. (Panels a, b) Monocytes infected with VB strain were stained with anti-EAV nsp1 MAb (panel a) or N MAb (panel b) and a specific antibody to cell surface antigen, CD14 MAb. (Panels c, d) Monocytes infected with MLV strain were stained with anti-EAV nsp1 MAb (panel c) or N MAb (panel d) and a specific antibody to cell surface antigen, CD14. (B) Replication of VB (black circles) and MLV (white circles) strains in purified monocytes. Tissue culture fluids were collected at indicated times and viral titers were determined as \( \log_{10} \) 50\% tissue culture infective dose (TCID\(_{50}\))/50\( \mu \)l. Values shown are the mean viral titers ± S.E.M. (C) Quantification of viral RNA copy number in cell culture fluid from purified monocytes infected with VB (black bars) and MLV (white bars) strains using qRT-PCR. Results are expressed as mean (± S.E.M) values.

**Figure 6. Infection of lymphocytes and monocytes with recombinant EAV viruses.**

The genome of the infectious full-length cDNA clone of rVBS (red boxes) and the genome of rMLV clone (blue boxes) are depicted. The genes encoding structural proteins of EAV HK116 virus are shown in green. The four chimeric viruses containing nonstructural and structural protein genes of either rVBS, or rMLV virus are also depicted. L, leader; An, poly A. The CD3\(^+\), CD4\(^+\) and CD8\(^+\) T lymphocytes and CD14\(^+\) monocytes infected with recombinant viruses rVBS (panels a through d), rVBS/HK116 S (panels e through h), rMLV (panels i through l), rVBS/MLV S (panels m through p), rMLV/VBS S (panels q through t), rMLV/VBS 234 (panels u through x) and rMLV/VBS 56 (panels y through z") were examined by dual-color immunofluorescence flow cytometric analysis using MAbs against EAV nsp1 (12A4) and MAbs for cell specific cell surface antigens at 24 hpi.

**Figure 7. Predicted membrane topology of minor (GP2, GP3 and GP4) and major (GP5 and M) EAV envelope proteins.** Amino acid substitutions occurred during extensive cell culture passage of the VB strain of EAV resulting in the HK116 and MLV strain of the virus are indicated. A predicted model for the disulfide-bonded structure of covalently
linked minor envelope proteins GP2, GP3 and GP4 proteins is depicted based on previous experimental studies (16, 25, 63-65). The major envelope GP5 and M proteins are covalently linked by a disulfide bond (S-S) formed between Cys-8 in the M protein and the Cys-34 in the GP5 protein (54).
Table 1. Comparative amino acid analysis of rVBS, HK116 and rMLV envelope proteins

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Note: Amino acid substitutions located in predicted ectodomain on each protein are indicated in bold. All potential N-glycosylation sites are conserved in GP2 (aa. 155), GP3 (aa. 28, 29, 49, 96, 106, 118) and GP4 (aa. 33, 55, 90).
A

Group A

% OF INFECTED CD3 T CELLS

HOURS POST INFECTION (HPI)

REAL FLOURESCENCE INTENSITY (α-EAV nsp1 AF488)

B

Group B

% OF INFECTED CD3 T CELLS

HOURS POST INFECTION (HPI)

REAL FLOURESCENCE INTENSITY (α-EAV nsp1 AF488)