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Growth Characteristics of a Highly Virulent, a Moderately Virulent, and an Avirulent Strain of Equine Arteritis Virus in Primary Equine Endothelial Cells Are Predictive of Their Virulence to Horses

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Equine viral arteritis (EVA) is an endotheliotropic viral disease of horses caused by equine arteritis virus (EAV). Although there is only one serotype of EAV, there is marked variation in the virulence of different strains of the virus. The replication and cytopathogenicity of three well-characterized strains of EAV of different virulence to horses were compared in rabbit kidney (RK-13) and primary equine pulmonary artery endothelial cells (ECs). Viral protein expression, plaque size, and cytopathogenicity of all three viruses were similar in RK-13 cells, whereas two virulent strains of EAV were readily distinguished from an avirulent strain by their plaque morphology and cytopathogenicity in primary equine ECs. Furthermore, EAV nucleocapsid protein was detected by flow cytometric analysis significantly later in ECs infected with the avirulent than those infected with the virulent strains of EAV. Primary equine ECs provide a convenient and relevant model for *in vitro* characterization of the pathogenesis of EVA and the virulence determinants of EAV.

Key Words: equine arteritis virus; equine endothelial cells; virulence.

INTRODUCTION

Equine arteritis virus (EAV) is the causative agent of equine viral arteritis (EVA), a reproductive and respiratory disease of horses (Timoney and McCollum, 1993). The virus was first isolated in 1953 from the lung of an aborted equine fetus during an abortion storm on a farm in Bucyrus, Ohio (Doll *et al.,* 1957). EAV is a positive-stranded RNA virus and is the prototype member of the genus *Arterivirus* in the family *Arteriviridae* of the order Nidovirales (Cavanagh, 1997). Other viruses in this genus include lactate dehydrogenase elevating virus of mice, simian hemorrhagic fever virus, and porcine reproductive and respiratory syndrome virus. There is only one serotype of EAV and all strains are neutralized by polyclonal antiserum to the virulent Bucyrus strain of EAV (Balasuriya *et al.,* 1997); however, geographically and temporally distinct strains of EAV differ in the severity of the disease they induce in horses and in their abortigenic potential (McCollum and Timoney, 1999; Patton *et al.,* 1998; Timoney and McCollum, 1993). The majority of field strains of EAV cause subclinical or inapparent infection in horses, whereas other strains cause the clinical manifestations of EVA (MacLachlan *et al.,* 1996; McCollum *et al.,* 1971; McCollum and Timoney, 1999; Timoney and McCollum, 1993). EAV principally replicates in macrophages and endothelium in infected horses, and the clinical manifestations of EVA reflect endothelial injury (Crawford and Henson, 1973; MacLachlan *et al.,* 1996). The characteristic vascular lesion of severe EVA is panvasculitis, with necrosis and leukocytic infiltration of medium and small muscular arteries (Crawford and Henson, 1973; Jones *et al.,* 1957; MacLachlan *et al.,* 1996). Although EAV infects endothelial cells, the mechanism of EAV-induced vascular injury is poorly characterized. Thus, in an effort to develop a cell culture system in which to further characterize the virulence determinants of EAV and the pathogenesis of EVA, we compared the replication and cytopathogenicity of virulent and avirulent strains of EAV in rabbit kidney (RK-13) and primary equine pulmonary artery endothelial cells (ECs).

RESULTS AND DISCUSSION

Pure cultures of primary equine pulmonary artery ECs with characteristic cobblestone morphology were isolated and propagated. Immunofluorescence staining for Factor VIII in perinuclear granules (Weibel-Palade bodies), uptake of 1,1′-dioctadecyl-3, 3,3′, 3′-tetramethylindocarbocyanine perchlorate conjugated to acetylated low-density lipoprotein (DiI-Ac-LDL), and lipopolysaccharide (LPS)-induced E-selectin expression established the identity of the cultured cells as ECs. The lack of any immunofluorescence staining with monoclonal antibodies (Mabs) to cytokeratin, smooth muscle actin, and

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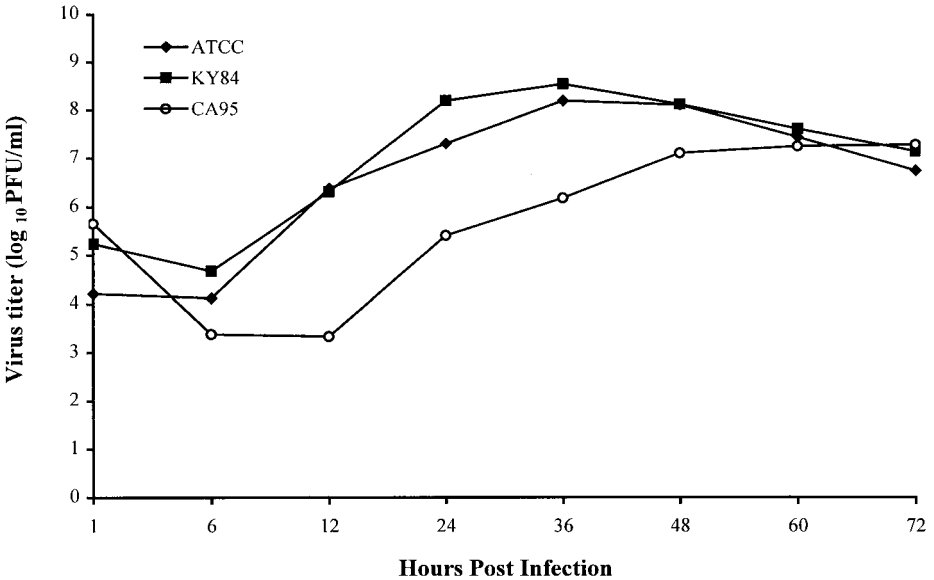

FIG. 1. Replication of an avirulent (CA95) and two virulent (ATCC, KY84) strains of EAV. Confluent monolayers of primary equine ECs were infected with partially purified preparations of each strain of EAV at an m.o.i. of 5.0 and virus titers (PFU/ml) were determined in sonicated whole cell lysates at the intervals after infection that are shown.

desmin confirmed the purity of the cultures. EC cultures were strongly contact inhibited, and maintained their phenotype and staining for Factor VIII for greater than 30 passages. All three strains of EAV (ATCC, Manasses, VA, KY84, and CA95) caused a lytic infection in both RK-13 and primary equine ECs; however, the onset and extent of cytopathic effect (CPE) varied markedly in EC cultures inoculated with the different viruses. The onset of CPE was especially delayed in EC cultures inoculated with the avirulent CA95 strain of EAV, and complete CPE never developed in these cultures. The ATCC and KY84 strains of EAV, which respectively cause severe and moderately severe EVA in horses, replicated more rapidly and to a higher titer in ECs than did the avirulent CA95 virus that causes inapparent infection of horses (Fig. 1). The ATCC and KY84 strains of EAV produced significantly larger plaques in ECs than did the avirulent CA95 strain ($P < 0.001$; Table 1, Figs. 2a and 2b), and the highly virulent ATCC strain of EAV also produced significantly larger plaques than did the moderately virulent KY84 strain ($P < 0.001$). In distinct contrast, there was no difference in the size of the plaques produced by any of these three strains of EAV in RK-13 cells.

The incidence of infection (percentage of infected cells) also was significantly different in EC cultures inoculated with the virulent and avirulent strains of EAV, as determined by expression of the EAV nucleocapsid (N) protein (Fig. 3). Specifically, the incidence of N-protein expression was significantly higher at 12, 24, 36, and 48 h postinfection (h.p.i.) in EC cultures infected with the highly virulent ATCC and moderately virulent KY84 strains than that in cultures infected with the avirulent

TABLE 1

Origin, Passage History, Plaque Morphology, and Virulence to Horses of Strains of EAV

Virus	Origin	Year isolated	Horse breed[a]	Cell type and passage history[b]	Plaque diameter (mm)		Severity of clinical disease in inoculated horses
					RK-13	EC	
ATCC	American Type Culture Collection	1953	STD	Eq 15, LLC-MK1, BHK 1, RK 2	3.58	3.67	Severe[c]
KY84	Kentucky	1984	TB	RK 3	3.46	2.31	Moderately severe[d]
CA95	California	1995	STD	RK 3	3.47	1.16	Subclinical[e]

[a] Horse breeds: TB, thoroughbred; STD, standardbred.

[b] Cells: RK, rabbit kidney-13; LLC-MK, rhesus monkey kidney cell line 2; BHK, baby hamster kidney 21; Eq 15, 15 serial passages in horses. Passage history: each cell type is followed by the number of passages in that cell type.

[c] Crawford and Henson (1973); Jones *et al.,* (1957); MacLachlan *et al.* (1996); McCollum *et al.* (1971).

[d] McCollum and Timoney (1984); Balasuriya *et al.* (2002).

[e] Patton *et al.* (1998).

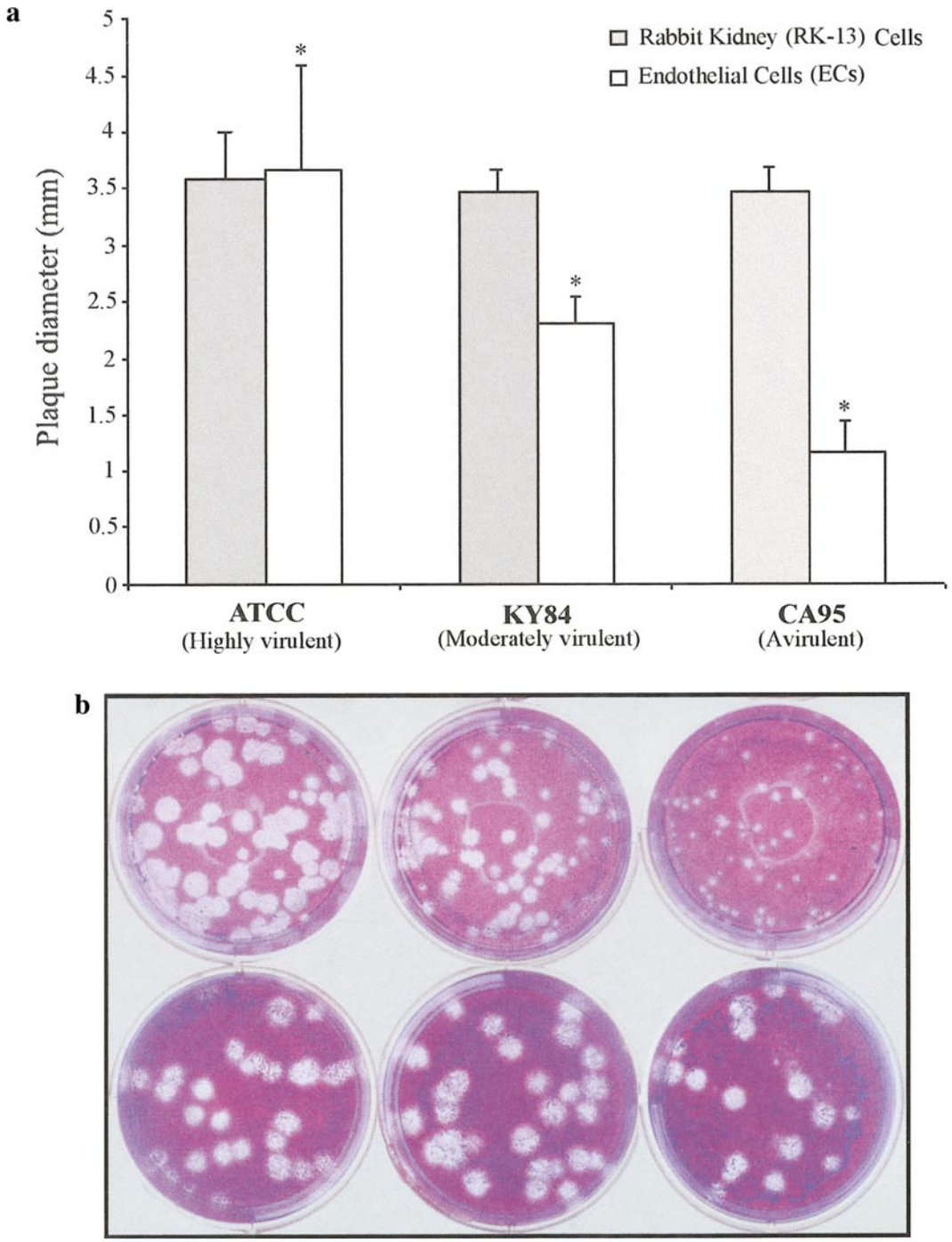

FIG. 2. (a) Size of plaques produced by the highly virulent ATCC, moderately virulent KY84, and avirulent CA95 strains of EAV in primary equine ECs and rabbit kidney (RK-13) cells. The vertical bars represent the mean ± standard deviation. An asterisk indicates a statistically significant difference ($P < 0.001$) in mean plaque diameter between the strains of EAV. (b) Plaque morphology of three strains of EAV in ECs (top row) and RK-13 (bottom row) cells.

CA95 strain ($P < 0.001$). There were no significant differences in the incidence of N-protein expression at any time interval after infection of RK-13 cells with these three strains of EAV.

Necrosis was the exclusive cause of cell death in both ECs and RK-13 cells infected with all three strains of EAV, and there were significant differences in the incidence of necrosis in EC cultures infected with the virulent and avirulent strains (Fig. 3). The highly virulent ATCC strain caused significantly more cytolysis at 36 ($P = 0.003$) and 48 ($P = 0.016$) hpi than did the moderately virulent KY84 strain. Similarly, both the highly virulent ATCC and moderately virulent KY84 viruses induced more cytolysis at 36, 48, and 72 h.p.i. ($P < 0.001$) than did the avirulent

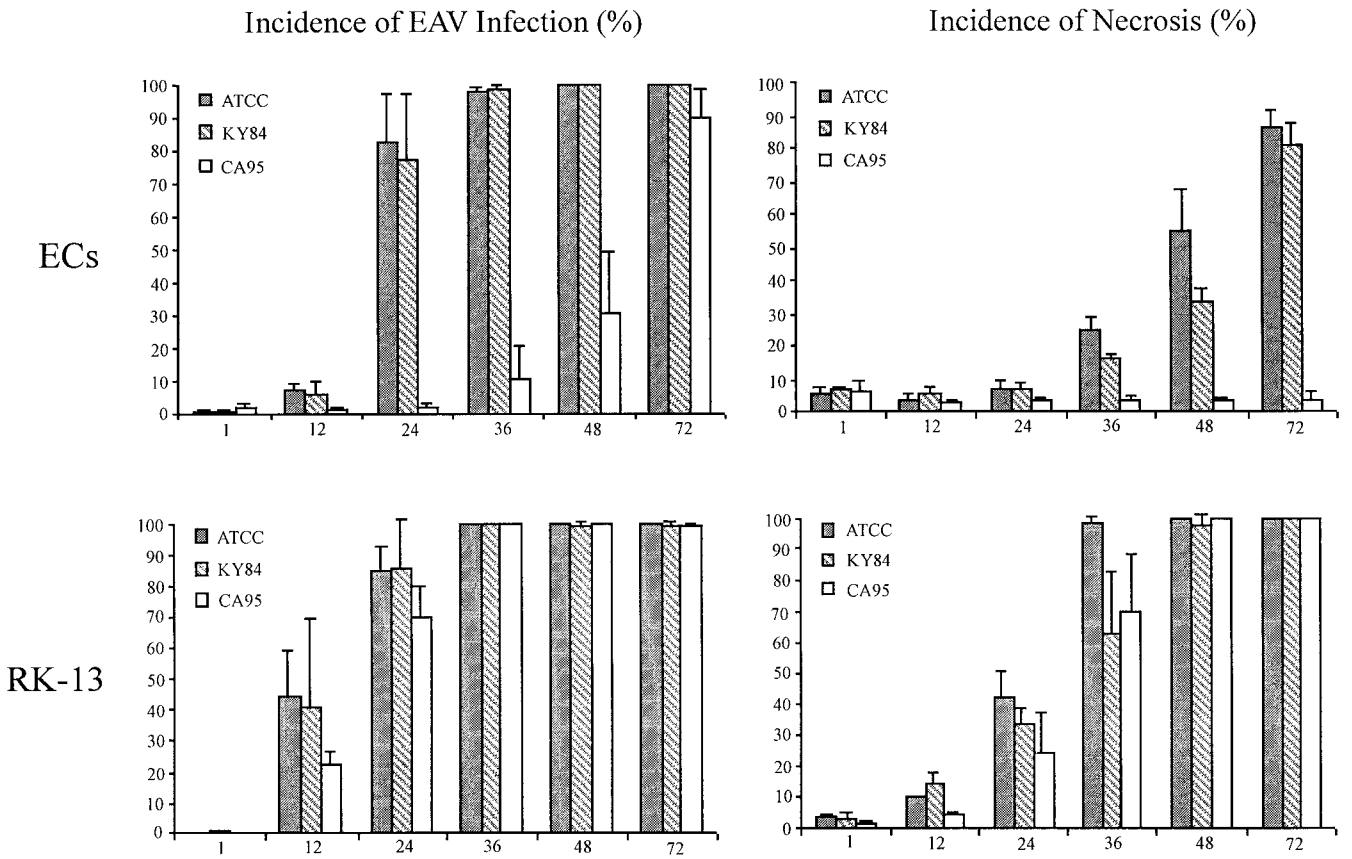

FIG. 3. Incidence (%) of infection and necrosis in primary equine ECs and rabbit kidney (RK-13) cells infected with the three strains of EAV, as determined by flow cytometric analysis. Vertical bars represent the mean ± standard deviation. Data were analyzed by repeated measures one-way ANOVA followed by *post-hoc* Tukey's multiple comparison. Significant differences in the percentage of infected cells between virulent (ATCC and KY84) and avirulent (CA95) strains of EAV occur at 12 ($P < 0.01$), 24 ($P < 0.001$), 36 ($P < 0.001$), and 48 ($P < 0.001$) h.p.i. Incidence of necrosis in ECs infected with the three strains of EAV as detected by propidium iodide exclusion (PI) and flow cytometric analysis significantly differs ($P < 0.001$) between virulent (ATCC and KY84) and avirulent (CA95) strains of EAV at 36, 48, and 72 h.p.i. There was no significant difference in the incidence of infection or necrosis in RK-13 cells infected with the virulent and avirulent strains of EAV.

CA95 virus. In contrast, the virulent and avirulent strains of EAV were not distinguished at any time interval after infection by the incidence of necrosis they induced in RK-13 cells.

Genetic variation and differences in virulence have been repeatedly demonstrated among field isolates and laboratory strains of EAV (Balasuriya *et al.,* 1995, 1998; Hedges *et al.,* 1996; McCollum and Timoney, 1999; Patton *et al.,* 1998). Thus, the objective of this study was to determine if the growth characteristics in primary equine ECs of three well-characterized strains of EAV were predictive of their virulence to horses. We clearly demonstrate, for the first time, differences in the replication and cytopathogenicity in equine ECs between EAV strains of different virulence to horses. Whereas replication of the different strains of EAV was not distinguished by plaque morphology, viral protein expression, or cytopathogenicity in RK-13 cells, the highly virulent ATCC, moderately virulent KY84, and avirulent CA95 strains of EAV were readily distinguished by their growth in equine ECs and this grouping precisely correlates to the virulence of each strain to horses (Balasuriya *et al.,* 2002; MacLachlan *et al.,* 1996; McCollum and Timoney, 1999; Patton *et al.,* 1998). Thus, the data confirm that differences in the virulence to horses of these strains of EAV may be predicted by *in vitro* assay in primary equine ECs.

In summary, whereas RK-13 cells provide a convenient and sensitive system for isolation and amplification of field strains of EAV (McCollum *et al.,* 1962), growth characteristics of different strains of EAV in this cell line are not predictive of their virulence to horses. In contrast, primary equine ECs provide a very relevant model for *in vitro* characterization of the virulence determinants of EAV and the pathogenesis of EVA.

MATERIALS AND METHODS

Cells and viruses

The isolation and purification of primary equine ECs using repeated fluorescence-activated cell sorting based on the uptake of DiI-Ac-LDL (Biomedical Technologies, Stoughton, MA) has been previously described in detail

(Hedges *et al.,* 2001). The purity of the EC cultures was confirmed by immunofluorescence staining with a panel of Mabs (DAKO Corp., Carpinteria, CA) that recognize epithelial and mesothelial cells (Mabs AE1/AE3, cytokeratin-specific), pericytes (Mab D33, desmin-specific), and smooth muscle cells (Mab 1A4, actin-specific). Antibody binding was detected with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Sigma, St. Louis, MO) by fluorescence microscopy and flow cytometric analysis using a FACScan instrument (Becton–Dickinson, San Jose, CA). The purified ECs were further characterized by immunofluorescence staining with rabbit antiserum to von Willebrand factor (Factor VIII; DAKO Corp.), the identification of Weibel-Palade bodies, which are highly characteristic of ECs, and by LPS-induced E-selectin expression (Hedges *et al.,* 2001). ECs were maintained in endothelial cell maintenance medium (Dulbecco's modified essential medium with sodium pyruvate; Mediatech, Herndon, VA), 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Inc., Logan, UT), antibiotics, nonessential amino acids, and 200 mM L-glutamine (Sigma). All experiments were performed on EC cultures between passages 5 and 15. RK-13 (ATCC CCL 37) cells were maintained in minimal essential medium with Earle's salts (EMEM) supplemented with 10% calf serum (Hyclone Laboratories, Inc.) and antibiotics.

The viruses compared in this study included the highly virulent (velogenic) ATCC strain of EAV (ATCC VR-796), which was derived by serial inoculation of horses with the original 1953 Bucyrus isolate of EAV; the moderately virulent KY84 strain of EAV that was isolated during an outbreak of EVA among thoroughbred horses in Kentucky in 1984; and the avirulent CA95 strain of EAV that was isolated from the semen of a persistently infected carrier standardbred stallion in California (Table 1). We and others have previously comprehensively defined the virulence to horses of each of the three strains of EAV used in this study (Balasuriya *et al.,* 2002; Crawford and Henson, 1973; Jones *et al.,* 1957; MacLachlan *et al.,* 1996; McCollum *et al.,* 1971; McCollum and Timoney, 1984, 1999; Patton *et al.,* 1998). Each virus was passaged once in RK-13 cells to prepare a partially purified stock. Briefly, RK-13 cells infected with each virus were frozen at −70°C after appearance of CPE. Cell lysates were clarified by low-speed centrifugation (1700 *g*), and the virus was pelleted by ultracentrifugation 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. Partially purified preparations of each strain of EAV were resuspended in EMEM and frozen at −70°C. The titer of each virus stock was determined as plaque-forming units (PFU)/ml in RK-13 cells. Similar preparations of uninfected RK-13 cells were prepared for mock infection of ECs.

Replication of EAV strains

The replication of the three strains of EAV was compared in equine ECs and RK-13 cells using plaque size, one-step growth curves, and infection rate (percentage of infected cells in each culture) as determined by expression of the N-protein of EAV. To determine plaque size, each virus was adsorbed for 1 h to confluent cultures of either ECs or RK-13 cells and the cultures were then overlaid with 0.75% carboxymethylcellulose (Sigma) containing maintenance medium. The monolayers were incubated for 96 h and fixed and stained with 1% crystal violet containing 1% formaldehyde. Replication of the three strains of EAV was compared in ECs that were infected with partially purified virus at a m.o.i. of 5.0 and incubated at 37°C. Individual cultures were harvested at 1, 6, and 12 and at subsequent 12-h intervals for 72 h.p.i. and titers of virus in sonicated culture lysates were determined by plaque assay on RK-13 cells essentially as previously described (McCollum *et al.,* 1962). Flow cytometric analysis after immunofluorescence staining for the N-protein of EAV was used to determine the percentage of EAV-infected cells in each culture. Briefly, ECs and RK-13 cells were infected with partially purified EAV at an m.o.i. of 5, and individual cultures were harvested at 12-h intervals after infection. Adherent cells remaining in each flask were removed by trypsinization, pooled with those previously detached from the media, and washed once with phosphate-buffered saline prior to fixation with 4% paraformaldehyde. Cells were then permeabilized with 0.1% saponin (Sigma) (Lavigne *et al.,* 1997). The presence of the N-protein of EAV was detected in infected cells by labeling with the N-protein-specific Mab 3E2 (MacLachlan *et al.,* 1996) followed by FITC-conjugated goat antimouse immunoglobulin (Sigma). Mock-infected cultures were harvested at 12, 24, 36, and 72 h for comparison.

Cell death in EC and RK-13 cultures infected with different strains of EAV

The incidence of cell death in EAV-infected EC and RK-13 cell cultures was quantitated by determining the percentage of apoptotic and/or necrotic cells in each culture at 12-h intervals after infection. Apoptosis and necrosis, respectively, were quantitated by double-label flow cytometric analysis based on the binding of annexin-V (R&D Systems, Minneapolis, NE) and the exclusion of propidium iodide (Sigma), as previously described (Vermes *et al.,* 1995). Forward light scatter and side light scatter characteristics on flow cytometric analysis also were used to determine the percentage of cytolysis in each culture and to further distinguish apoptotic from necrotic cells (Darzynkiewicz, 1997).

Data analysis

Data were analyzed with the CELLQuest version 3.1 (Becton–Dickinson, San Jose, CA) and MINITAB (Minitab Inc., State College, PA) software packages. All experiments were repeated at least five times and the data from replicate experiments were compared at 1, 12, 24, 36, 48, and 72 h.p.i. Mean values and standard deviations were calculated for each parameter with a repeated measures one-way ANOVA (parametric analysis of population means) to determine differences between mean values of the data at each time point. Differences were considered significant at $P \leq 0.05$. If the overall ANOVA was significant, a *post-hoc* Tukey's multiple comparison of the mean was performed on data at each time point to evaluate differences between the various EAV strains.

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