Human cytomegalovirus (HCMV) establishes a lifelong persistence in the host after primary infection. Although macrophages from the peripheral blood have recently been identified as a site of HCMV latency in asymptomatically infected individuals (21), another potential cell type latent or persistently infected by HCMV is vascular endothelial cells (EC). Studies of autopsy tissue from HCMV-seropositive transplant patients have revealed that EC commonly harbor virus without obvious cytopathology (14). However, additional studies have indicated that EC constitute one of several cell types that exhibit cytopathology (14). An understanding of the role of EC in HCMV disease has been complicated by the detection of viral DNA in arterial specimens from seropositive individuals without active infection (12). These observations have led to the speculation that EC may be a virus reservoir.

EC exhibit phenotypic differences that are dependent on the origin (adult versus fetal), anatomical location, and vessel size (large vessel versus capillary) (16, 22). Human umbilical vein EC (HUVEC), which are commonly utilized for EC studies, are derived from fetal large-vessel tissue. These cells are functionally and biochemically distinct from EC derived from adult tissue such as large-vessel aortic EC (AEC) (16, 22). Capillary EC not only display unique differences from large-vessel EC but also demonstrate organ specificity. For example, human brain microvascular EC (BMVEC), which together with astrocytes compose the brain-blood barrier, possess specific transporter systems that regulate the passage of specific metabolites from the blood to the brain parenchyma (8, 13). These unique properties differentiate BMVEC from EC in capillaries of other tissues. The physiological and biochemical differences between EC in different organs may affect the ability of HCMV to replicate in these cells.

HCMV infection of EC in vitro has been controversial. Early studies suggested that HCMV was unable to replicate in EC (4). However, others suggested that the virus could productively infect a low percentage of cells in culture (20). In addition, HCMV infectivity of EC was enhanced by serial passage of virus through these cells. Interestingly, viral infection of HUVEC resulted in anchorage-independent growth and a transformed phenotype (23). An important consideration, however, is that viral replication in HUVEC may not represent viral replication in adult EC. In support of this hypothesis, other viruses have demonstrated exquisite cellular specificity concerning their abilities to productively infect EC obtained from different organs (13). Lathey et al. addressed this issue when they demonstrated that HCMV infected BMVEC more efficiently than HUVEC, suggesting that the observed physiological differences between EC types may also affect viral replication (10).

In the present study, we examined characteristics of HCMV replication in AEC. We found that HCMV infection was not lytic and resulted in the accumulation of significant amounts of extracellular but not intracellular virus. In addition, the cell cycle was not inhibited by HCMV and cells continuously released infectious virus. These results contrast with those showing the rapid, lytic infection of BMVEC and HF cells. The ability of HCMV to infect AEC and to continuously produce extracellular virus and the absence of cytopathic effect are prerequisites for establishing viral persistence. Therefore, HCMV infection of AEC provides an ideal model to examine mechanisms of persistence in the human host.

**MATERIALS AND METHODS**

Culturing and infection of EC. BMVEC were a generous gift from Ashlee Moses (Oregon Health Sciences University, Portland, Ore.), and AEC were purchased from Clonetics Normal Human Cell Systems (San Diego, Calif.).

**Endothelial cells (EC) have been implicated as constituting an important cell type in the pathogenesis of human cytomegalovirus (HCMV). Microvascular and macrovascular EC exhibit different biochemical and functional properties depending on the organ of origin. Phenotypic differences between microvascular and macrovascular EC may alter the ability of these cells to support HCMV replication. In this study, we compared the replication of HCMV in primary macrovascular aortic EC (AEC) with that in brain microvascular EC (BMVEC). An examination of IE72, pp65, and gB viral antigen expression in BMVEC and AEC by immunofluorescence revealed similar frequencies of infected cells. Intracellular production of virus was 3 log units greater in BMVEC than in AEC, while equal quantities of extracellular virus were produced in both cell types. HCMV infection of BMVEC resulted in rapid cellular lysis, while the virus was nonlytic and continuously released from HCMV-infected AEC for the life span of the culture. An examination of infected cells by electron microscopy revealed the formation of abundant nucleocapsids in both AEC and BMVEC. However, significant amounts of mature viral particles were only detected in the cytoplasm of BMVEC. These observations indicate that levels of HCMV replication in EC obtained from different organs are distinct and suggest that persistently infected AEC may serve as a reservoir of virus.
BMVEC were cultured in Endo-SFM medium (GBCO Laboratories, Grand Island, N.Y.), containing 10% human AB serum (Sigma Chemical Co., St. Louis, Mo.), 1.0% penicillin-streptomycin solution (GBCO), 1.0% glutamine (Sigma), heparin (40 μg/ml; Sigma), and EC growth factor (50 μg/ml; Sigma). AEC were cultured in the media recommended by the manufacturer. Since BMVEC and AEC cultures represented single donors, each experiment was repeated in triplicate with different donors. Low-passage-number (less than 5) BMVEC or AEC were plated in 35-mm² Primaria culture dishes (Becton Dickinson, Lincoln Park, N.J.) or two-well coverslip bottom Lab-Tek chamber slides (Nunc, Inc., Naperville, Ill.) and allowed to grow at 37°C with 7% CO₂ to 70% confluency prior to infection with the HCMV laboratory strain Towne, a recent patient isolate Po (3), or the mutant laboratory strain AD169 pp65(17). Heparin-free medium was used at least 1 h prior to infection and throughout the infection time course. Postinfected EC cultures were fixed in 1% osmium tetroxide in the same buffer for 1 h at 4°C, dehydrated in cacodylate buffer (pH 7.4) at 4°C for 16 to 20 h. Fixed cells were collected by electron microscopy (EM), uninfected and infected cells were harvested at 3, FACS scan instrument was used for analyzing the stained nuclei. Fluorescence-assisted cell sorting (FACS) analysis. Becton Dickinson Cell Quest was added, and the samples were incubated for 30 to 60 min at 4°C prior to adding serum to the medium. Since BMVEC and AEC were fixed at various intervals postinfection and examined for the presence of the HCMV IE antigen and either the lower-matrix phosphoprotein pp65 or the major envelope glycoprotein gB by double-label immunofluorescence (Fig. 2). By 3 dpi, 80% of the AEC and 50% of the BMVEC displayed the presence of HCMV antigens. Although the frequency of IE antigen detection was greater in AEC, expression was delayed in these cells, with the first appearance of antigen at 24 h postinfection (hpi) in contrast to 12 hpi in BMVEC (Fig. 2A and B [red nuclei]). A minor delay in the expression of pp65 and gB was also observed in AEC, in comparison to their expression in BMVEC. An examination of gB expression in BMVEC revealed the presence of antigen in intracellular vacuoles and at the plasma membrane (PM) between 2 and 3 dpi (Fig. 2C [green and blue fluorescence, respectively]). In contrast, gB was detected in infected AEC at 3 dpi in intracellular vacuoles but not at the PM (Fig. 2C [green fluorescence]). These results demonstrate that the frequency of infected cells, the kinetics of viral antigen expression, and the distribution of viral antigens within the cell differ between HCMV-infected BMVEC and AEC.

RESULTS

HCMV is lytic in BMVEC but not AEC. Since AEC are naturally infected in vivo and represent a potential reservoir of persistent virus, we examined the ability of HCMV to infect primary cultures of these large-vessel EC in vivo compared to the ability of the virus to infect BMVEC. The EC cultures were >95% pure as determined by the presence of vWF (Fig. 1A). AEC and BMVEC subconfluent monolayers were infected with HCMV (Towne) at an MOI of 3 and examined at 3, 8, and 14 days postinfection (dpi) by phase microscopy. HCMV infection of BMVEC resulted in the development of cytopathic effect by 5 dpi and in lysis of 70% of the cells in culture at 14 dpi (Fig. 1B and data not shown). Surprisingly, a cytopathic effect was not observed in AEC infected with HCMV up to 30 dpi, the in vitro life expectancy of these cells (Fig. 1B and 2B and data not shown).

To determine the frequency of HCMV infection, BMVEC and AEC were fixed at various intervals postinfection and examined for the presence of the HCMV IE antigen and either the lower-matrix phosphoprotein pp65 or the major envelope glycoprotein gB by double-label immunofluorescence (Fig. 2). By 3 dpi, 80% of the AEC and 50% of the BMVEC displayed the presence of HCMV antigens. Although the frequency of IE antigen detection was greater in AEC, expression was delayed in these cells, with the first appearance of antigen at 24 h postinfection (hpi) in contrast to 12 hpi in BMVEC (Fig. 2A and B [red nuclei]). A minor delay in the expression of pp65 and gB was also observed in AEC, in comparison to their expression in BMVEC. An examination of gB expression in BMVEC revealed the presence of antigen in intracellular vacuoles and at the plasma membrane (PM) between 2 and 3 dpi (Fig. 2C [green and blue fluorescence, respectively]). In contrast, gB was detected in infected AEC at 3 dpi in intracellular vacuoles but not at the PM (Fig. 2C [green fluorescence]). These results demonstrate that the frequency of infected cells, the kinetics of viral antigen expression, and the distribution of viral antigens within the cell differ between HCMV-infected BMVEC and AEC.
FIG. 1. HCMV cytopathic effect in BMVEC and AEC. To ensure the purity of EC, AEC and BMVEC were stained for the presence of vWF. As shown in panel A, >95% of the cells displayed the presence of vWF (magnification, ×125). Panel B demonstrates phase microscopy of HCMV-infected AEC and BMVEC and HF cells at the indicated intervals postinfection. As shown in this panel, cytopathic effect is observed by 8 dpi in BMVEC but not in AEC throughout the time course of infection (magnification, ×50).
FIG. 2. Expression of HCMV antigens in infected AEC and BMVEC. (A) HCMV-infected AEC and BMVEC were examined by immunofluorescence to assess the frequency of infected cells as well as the distribution of viral antigens within the cell. Quantitation of the number of infected cells indicated that at least 80% of the AEC and 50% of the BMVEC expressed an HCMV antigen (IE72 or pp65). (B) To determine the frequency of HCMV infection, double-label immunofluorescence was performed with antibodies directed against the IE72 antigen (rhodamine; red) and the pp65 antigen (fluorescein; green). Magnification, ×63. (C) To determine the subcellular location of viral antigens, double- and triple-label immunofluorescence was performed with antibodies directed against the IE72 antigen (rhodamine; red), pp65 (BMVEC at 1 dpi as indicated by the arrow; fluorescein; green), or intracellular gB antigen (fluorescein; green), and cell surface gB antigen (BMVEC at 3 dpi as indicated by the arrow; cyanine-5; blue). Magnification, ×265.
amounts of mature virions were observed in the cytoplasm of infected AEC. These results are consistent with the inability to detect large quantities of infectious virus within AEC.

**HCMV infection of AEC does not inhibit the cell cycle.** The ability of HCMV to nonlytically infect AEC and to produce infectious virus suggests that these cells may be a persistent reservoir of virus. HCMV-infected AEC did not differ in their phenotypic properties from mock-infected cells for up to 30 dpi in culture or when passaged (Fig. 6A and data not shown). The latter result suggests that HCMV does not inhibit the ability of AEC to progress through the cell cycle. To address this issue, subconfluent AEC cultures were serum starved for 48 h, followed by HCMV infection at an MOI of 5. At this MOI greater than 95% of the cells were positive by FACS for the IE antigen (data not shown). The ratio of infected to uninfected cells was similar up to 5 dpi (Fig. 6A). These observations indicate that HCMV does not alter the frequency of cell division in infected cells.

The above results contradict previously published observations that HCMV infection of HF cells inhibits entry of cells into G2 (2, 7, 11). A series of experiments was designed to examine this apparent discrepancy. To synchronize the cell culture at G0, AEC and HF cultures were serum starved for 72 h. Serum was then added 3 h prior to infection with HCMV. AEC and HF cultures were harvested, and nuclei were prepared at different intervals up to 5 dpi. Nuclei from infected and mock-infected cultures were stained with propidium iodide either alone or in combination with an IE-specific antibody, followed by FACS analysis (Fig. 6B and data not shown). In contrast to the inhibition of the cell cycle in HCMV-infected HF cells (data not shown) (2, 7, 11), the numbers of nuclei in G2 in both the mock-infected and the HCMV-infected AEC cultures remained comparable up to 5 dpi. In addition, spindle poles were commonly detected in HCMV-infected AEC, which is consistent with the observation that HCMV does not inhibit cell division (Fig. 6C and D). These results support the hypothesis that HCMV does not block the AEC cell cycle.

**DISCUSSION**

In this study we demonstrate that EC obtained from different adult tissues respond differentially to HCMV infection. Although both BMVEC and AEC are productively infected by virus, infection of BMVEC resulted in the rapid lysis of cells, while infected AEC cultures exhibited a sustained noncytopathic infection for over 30 dpi. Productive infection in the absence of cytopathology is a prerequisite for the establishment of persistence. Since HCMV infection of AEC in vitro is noncytopathic and since AEC are infected in vivo, this observation suggests that the large-vessel endothelium may be an HCMV reservoir in vivo.

The mechanism by which HCMV induces a persistent noncytopathic infection of AEC is unknown. HCMV-infected AEC were unable to accumulate infectious intracellular virus, although they produced normal amounts of extracellular virus. This phenomenon suggests that the cells survive infection through efficient export of mature virus and toxic products, which may cause cellular lysis, from the cell. This mechanism is clearly different from that which operates during HCMV infection of monocyte-derived macrophages, which results in the nonlytic accumulation of large amounts of intracellular virus and the lack of extracellular virus (3). In monocyte-derived macrophages, virus was found to accumulate in large cytoplasmic vacuoles that did not associate with the PM. These two observations emphasize the cell-specific differences that occur during HCMV infection.

In addition to the extremely rapid export of virus from AEC, the inability of HCMV to block the cell cycle may be a way in which the virus and cell coexist. The mechanism by which the cell or virus or both circumvent the normal blocks in the cell cycle due to infection is unknown. Jault et al. (7) observed an...
overexpression of cyclin E and a delay in cyclin A accumulation in HCMV-infected cells. Cyclin A is required for DNA replication and for the G2/M transition (5, 15). A proposed mechanism by which HCMV affected cyclin A accumulation was dependent upon viral and cellular DNA replication kinetics. Therefore, one might speculate that viral and cellular DNA replication kinetics in HCMV-infected AEC are such that the viral cell cycle inhibitors are not present at the appropriate times during the cell cycle. As the complexities of how HCMV affects the cell cycle are discovered, the mechanism by which AEC elude a block in the cell cycle may be revealed.

Although the specific cellular organs HCMV targets during acute disease have been known for almost 50 years, the site(s) for HCMV latency has been difficult to identify. We have recently demonstrated that latent HCMV can be reactivated in a myeloid lineage cell obtained from the peripheral blood of healthy seropositive individuals (21). However, this observation does not preclude the existence of other sites of HCMV latency or persistence. Another likely cellular candidate for HCMV persistence is the EC. AEC interact naturally with monocytes that are trafficking in the bloodstream and migrating into tissues. Therefore, a dynamic interaction between the cells in which virus is either activated or transmitted in tissues may occur during extravasation. These interactions may involve cell-cell contact through adhesion molecules, which subsequently triggers signal transduction events, activating virus in latently infected monocytes or the resting endothelium. The monocyte or AEC may function as a vehicle for cell-to-cell transmission of HCMV, which in turn could result in the reactivation of HCMV from macrophages or EC in the artery. One consequence of HCMV reactivation in the artery is the dissemination of virus throughout the body. HCMV would be easily spread throughout the circulatory system once shed into the lumen of the aorta because of the large quantities of blood that pass through this organ. The state of viremia may then result in the widespread infection of different organs. In addition to causing the dissemination of free virus throughout the body, circulating monocytes that become infected or that reactivate virus because of contact with AEC would serve as the ideal vector for HCMV dissemination to other tissues. In support of this hypothesis, an in vitro model has demonstrated that infected EC can transmit HCMV to cocultured monocytes.

FIG. 5. HCMV-infected AEC and BMVEC were infected with HCMV at an MOI of 3 and analyzed by EM at 14 dpi. Small amounts of cytoplasmic virus were detectable in AEC when they were compared to BMVEC by EM (A & C; bar = 2 μm). Similar amounts of nucleocapsids were detected in the nuclei of AEC and BMVEC by EM (B and D; bar = 350 nm). The arrows in panels B and D show examples of mature nucleocapsids.
FIG. 6. HCMV infection of AEC does not result in the arrest of the cell cycle. AEC were infected at an MOI of 5 or were mock infected. The culture doubling frequency was determined throughout a time course of infection (A). Nuclei from infected and mock-infected AEC were stained with propidium iodide alone or in combination with IE antibody and used for FACS analysis (B). AEC were infected with HCMV, and at 5 dpi double-label immunofluorescence was performed with antibodies directed against the IE72 antigen (C) and microtubulin (D). Magnification (C and D), ×407. The arrow in panel D points to spindle poles.
and that these monocytes can retransmit virus to uninfected EC (23).

This study clearly indicates the importance of EC in the biology of HCMV. Elucidating the mechanisms of HCMV replication and virus assembly in AEC will be essential for understanding viral persistence and trafficking in the human host. The current studies indicate that HCMV replication in AEC (23).

ACKNOWLEDGMENTS

We thank Rebecca Ruhl for technical assistance and Ashlee Moses for helpful discussion.

This work was supported by a Public Health Service grant from the National Institutes of Health (AI 21640 (J.A.N.), the Molecular Hematology Training program NIH NRSA Training Award (K.N.F.), and the Knut and Alice Wallenberg Foundation (C.S.-N.). C.S.-N. is a scholar of the Wenner-Gren Foundation, Sweden.

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