Chronic infection of human umbilical vein endothelial cells by human herpesvirus-6

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Human herpesvirus-6 (HHV-6) exhibits a predominant tropism for CD4+ T-lymphocytes, but can infect other components of the blood as well as surrounding tissue and organs. To understand the role of the endothelium in the transmission and haematogenous spread of this virus, human umbilical vein endothelial cells (HUVEC) were infected with HHV-6 and monitored for viral gene expression. The presence of both early and late viral antigens was demonstrated by indirect immunofluorescence in 37 ± 6 and 6 ± 5%, respectively, of HUVEC. However, attempts to detect the release of infectious virus were not successful, indicating infection is semi-permissive in nature. Upon continued passage of infected HUVEC monolayers, HHV-6 antigen-positive cells persisted up to 27 days post-infection. Furthermore, the virus could be recovered from HUVEC monolayers that contained fewer than 1% antigen-positive cells by co-cultivation with peripheral blood mononuclear cells. Together, these findings suggest that endothelial cells may serve as a reservoir for harbouring HHV-6.

Introduction

Human herpesvirus-6 (HHV-6) was first isolated in 1986 from the blood of six patients with lymphoproliferative disorders, two of whom also presented with AIDS (Salahuddin et al., 1986). HHV-6 has been demonstrated to be the causative agent of the childhood disease exanthem subitum, infecting over 90% of the population by the age of 2 years (Yamanishi et al., 1988). This illness is characterized by a high-grade fever, often followed by the appearance of a rash, and in most cases the symptoms generally resolve within 3–6 days. However, neurological complications have been reported and primary HHV-6 infection is responsible for approximately one-third of febrile seizures observed in children (Hall et al., 1994).

Like other herpesviruses, HHV-6 is maintained in a latent state after primary infection. Reactivation of HHV-6 poses a serious health problem for immunocompromised individuals, particularly patients undergoing bone marrow transplantations and victims of AIDS (Drobsky et al., 1993; Carrigan & Knox, 1994; Lusso & Gallo, 1995). Here, the virus has been demonstrated to be an opportunistic pathogen and in some cases is responsible for causing fatal pneumonitis (Carrigan et al., 1991; Cone et al., 1993; Knox et al., 1995). Autopsy reports of AIDS patients have documented widespread dissemination of HHV-6, detecting the virus in the lung, spleen, kidney, lymph system and liver (Corbellino et al., 1993; Knox & Carrigan, 1994).

The pathogenesis of HHV-6 infection is poorly understood. While HHV-6 exhibits a predominant tropism for CD4+ T-lymphocytes, the virus has been shown to infect a number of other components of blood including monocytes/macrophages and B-lymphocytes (Ablashi et al., 1987; Lusso et al., 1988; Takahashi et al., 1989; Kondo et al., 1991). Moreover, while dissemination of the virus to multiple tissues has been observed in both immunocompromised and immunocompetent patients (Knox & Carrigan, 1994; Prezioso et al., 1992), the mechanism of haematogenous spread is unknown. Endothelial cells lining the walls of blood vessels serve as an important barrier between components of the blood and surrounding tissues of the body. This study was designed to investigate whether HHV-6-infected T-lymphocytes could transfer infection to endothelial cells, providing a means of transmission and dissemination of the virus. Endothelial cell cultures were observed to support both HHV-6 early and late gene expression. Furthermore, the virus persists within these cells at a low frequency for several weeks, suggesting that the endothelium may serve as a reservoir for HHV-6.

Methods

Cells and virus. HHV-6A isolate U102 (gift of Frank Jenkins, University of Pittsburgh, PA; originally isolated by Robert Honess, National Institute for Medical Research, UK) and HHV-6B isolate R255

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BH6-6 cell-free virus stocks were prepared by collecting the supernatants from BH6-6-infected Jhan cell cultures showing evidence of > 70% infection by indirect IF. Such cell-free inoculum routinely had a titre of $10^7$ TCID$_{50}$/ml. HUVEC (1 × 10$^5$ cells per well) were seeded on glass coverslips, maintained to 80% confluence, and overlaid with 2 × 10$^6$ BH6-6-infected Jhan cells (23–50% positive for BH6-6 antigen by indirect IF) or infected with 250 TCID$_{50}$ cell-free inoculum. After 24 h, HUVEC monolayers were washed three times with ECBM and incubation continued in fresh ECBM.

### HHV-6 early and late gene expression.

To monitor HHV-6 infection, HUVEC were analysed for early and late viral gene expression as follows. Infected cells were fixed for 20 min at room temperature in a freshly prepared solution of 3.7% formalin in PBS, washed three times in PBS, and permeabilized by exposure to 0.5% Triton X-100 in PBS for 5 min. Cells were incubated at room temperature for 1 h in the presence of HHV-6 specific monoclonal antibodies (MABs), washed in PBS, and incubated for 1 h with goat anti-mouse IgG conjugated with rhodamine (Organon Teknika). The coverslips were mounted in glycerol–veronal saline pH 8.6 (25 mM, 5.5-diethylbarbituric acid, 50 mM NaCl, 50% glycerol) in preparation for phase-contrast and fluorescence analysis using a Leitz Diaplans microscope. MAB p41 (Virotech International) is specific for the DNA polymerase accessory protein (ORF U27) from both HHV-6A and B glycoprotein 102 (ORF U48; glycoprotein H), HHV-6A and B glycoprotein 16 (ORF U39; glycoprotein B), and HHV-6A glycoprotein complex 82/103 (ORF U100), respectively (gifts of Bala Chandran, University of Kansas Medical Center, KS), and serve as markers for early gene expression (Chang & Balachandran, 1991). MABs 7A2, 6A5G3 and 2D6 recognize HHV-6-A and B antigen for 10 days. Cells were centrifuged onto glass coverslips using a Shandon cytosin device and analysed for HHV-6 early gene expression by indirect IF using HHV-6 MAB p41 as described above.

### Production of infectious virions.

HUVEC were grown to 80% confluency on a six-well plate and inoculated with a cell-free HHV-6 stock at $10^5$ TCID$_{50}$/ml. On days 6, 8, 10 and 12 after infection, HUVEC culture supernatants were harvested and clarified by centrifugation, and 250 μl used to inoculate 4 × $10^5$ CBMC grown in RPMI 1640 supplemented with 10% foetal bovine serum, 10 ng/ml hydrocortisone and 32 units/ml interleukin-2. CBMC cultures were incubated at 37°C for 10 days. Cells were centrifuged onto glass coverslips using a Shandon cytosin device and analysed for HHV-6 early gene expression by indirect IF using HHV-6 MAB p41 as described above.

### Propagation of HHV-6 in HUVEC.

HUVEC were examined for their ability to maintain HHV-6 infection. Specifically, T-25 flask of HUVEC were infected with HHV-6-A cell-free inoculum, as described above. Upon reaching confluency, the HUVEC monolayers were washed with PBS, treated with trypsin, and collected by centrifugation. One-third of the resulting cell pellet was maintained in culture, one-third was seeded onto a coverslip and fixed within 6 h for indirect IF, and the remaining one-third was discarded. This pattern of propagation was continued approximately every 5 days for 5–6 weeks and resulted in HUVEC monolayers up to passage 14. When no HHV-6 gene expression was detected (as measured by indirect IF with p41 antibody) for two passages, HUVEC monolayers were maintained untreated; treated with 3 mM sodium butyrate; or treated with 12-O-tetradecanoylphorbol 13-acetate (TPA) at 14.3 ng/ml. After 24 h, the medium was removed and HUVEC monolayers were co-cultured with 1 × $10^5$ CBMC for 6 h. The CBMC were removed and maintained in a separate T-25 flask, while the HUVEC were incubated in fresh ECBM. Both HUVEC and CBMC were assayed for HHV-6 early gene expression by indirect IF 4 and 10 days, respectively, after co-cultivation.

### Pretreatment of HUVEC with cytokines.

To determine the effect of various mediators and cytokines on HHV-6 infection of endothelial cells, HUVEC were seeded onto coverslips and pretreated 24 h prior to
infection with lipopolysaccharide (Sigma), recombinant tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)) (Genentech), recombinant interleukin 1\(\alpha\) (IL-1\(\alpha\)) (Genzyme Diagnostics), recombinant interferon \(\alpha\)2b (IFN-\(\alpha\)) (Schering), or recombinant interferon-\(\gamma\) (IFN-\(\gamma\)) (Genzyme Diagnostics) at the indicated concentrations. After co-cultivation for 24 h with \(2 \times 10^5\) HHV-6A-infected JJhan cells, the HUVEC monolayers were washed three times and incubated with medium in the absence of cytokines. Three days after infection, indirect IF staining for HHV-6 early gene expression was performed with MAb p41, as described above.

**Results**

**HHV-6 early and late gene expression in endothelial cells**

To determine the susceptibility of endothelial cells to HHV-6, monolayers of HUVEC were overlaid with HHV-6A isolate U1102-infected JJhan cells. During the course of infection enlarged, multi-nucleated cells were observed by phase microscopy in infected HUVEC cultures (Fig. 1C). These foci appeared within 24 h, and were not present in HUVEC exposed to uninfected JJhan cells (Fig. 1A).

Early and late viral gene expression was detected in HHV-6A-infected HUVEC monolayers by indirect IF 3 days after infection. Using a MAb specific for the DNA polymerase accessory protein (antibody p41), early viral antigen was detected in infected HUVEC nuclei. The staining pattern observed was both diffuse throughout the nucleus and a distinct punctate design (Fig. 1D). Late gene expression was monitored with a MAb against the glycoprotein complex 82–105 (antibody 2D6). In this case, staining was cytoplasmic in nature (Fig. 1F). Multi-nucleated syncytia, as well as individual cells within the HUVEC monolayer, stained positive for both early and late HHV-6 proteins. HUVEC monolayers co-cultured with uninfected JJhan cells exhibited no nuclear (Fig. 1B) or cytoplasmic (data not shown) fluorescence with either antibody.

Susceptibility of endothelial cells to viral infection was not limited to an HHV-6A variant. Co-cultivation of HUVEC monolayers with HHV-6B isolate R255-infected JJhan cells resulted in the production of enlarged, multi-nucleated foci as described above. Both early and late gene expression was detected using MAbs p41 and 7A2 (glycoprotein 102), respectively (Fig. 1H, J). In contrast to the pattern of early antibody staining observed with HHV-6A, HHV-6B-infected HUVEC exhibited only a punctate pattern (Fig. 1, compare D and H).

**Phenotypic identification of HHV-6-infected cells**

In the experiments described above, HHV-6-infected JJhan cells were used as the source of infectious material. The majority (> 90%) of both infected and uninfected JJhan cells were removed from the endothelial cell culture by extensive washing, and the remaining JJhan cells were morphologically distinct from the HUVEC monolayer. However, to confirm the phenotypic identity of HHV-6 antigen-positive cells, infected HUVEC were dually stained with HHV-6 p41 antibody and a MAb specific for von Willebrand factor (factor VIII), a glycoprotein synthesized by endothelial cells (Wagner, 1990). Both individual HUVEC and multi-nucleated syncytia, characteristic of HHV-6 infection, expressed von Willebrand factor as demonstrated by immunohistochemical cytoplasmic staining.
in purple (Fig. 2B, C). Several of the endothelial cells also exhibited a brown nuclear stain, indicative of HHV-6 early antigen. It is interesting to note in Fig. 2(C) that only two out of four HUVEC nuclei present in the syncytium are positive for HHV-6 early antigen. As a control, endothelial cells were co-cultured with uninfected JJhan cells and dually stained as described above. Such cultures were uniformly positive for von Willebrand factor, exhibiting a strong cytoplasmic staining pattern (Fig. 2A), but lacked nuclear staining, denoting an absence of HHV-6 early antigen.

**Time-course of HHV-6 infection**

HHV-6A early and late gene expression in HUVEC was monitored over time using the indirect IF assay described in Methods. Four independent experiments were performed and the data from two representative experiments are shown in Fig. 3. HUVEC, co-incubated with HHV-6A-infected JJhan cells, exhibited early viral gene expression 24 h after infection (Fig. 3A). The number of HHV-6 p41 positive cells increased with time, peaking between days 3 and 4 post infection (p.i.), with a maximum of 37-6% of the monolayer staining positive for the virus. The synthesis of HHV-6 late proteins was first noted on day 2 after infection, and the frequency of HUVEC expressing HHV-6 late antigens increased only slightly with time from 2-0% on day 2 to 6-4% on day 4. This ratio of cells synthesizing early versus late gene products, approximately 5 to 1, was consistently observed in four separate experiments. No IF staining was observed in HUVEC co-incubated with uninfected JJhan cells using antibodies specific for either the HHV-6 early or late gene products.

HHV-6A early and late gene expression was also examined in HUVEC using cell-free virus inoculum (Fig. 3B). The number of cells expressing HHV-6 early antigen increased gradually from day 1 to day 5 after infection, reaching a maximum level of 29-2%. Expression of HHV-6 late gene products was first detected on day 2 and reached a plateau of 7-2% on day 4. These results depict a similar ratio of early to late gene expression as compared with experiments using HHV-6-infected JJhan cells as the inoculum.

The total number of HUVEC present in infected cultures was monitored over time and compared with the number of endothelial cells present in mock-infected cultures at the same time point. The results from the experiments in Fig. 3(A, B) were calculated as a percentage of control and summarized in Fig. 3(C). When using either HHV-6-infected JJhan cells or cell-free virus as the inoculum, only 40% of the HUVEC monolayer remained intact at 5 days p.i. This pronounced decrease in the number of HUVEC present in culture suggests that HHV-6 infection leads to destruction of the endothelium.

**Replication of HHV-6 DNA**

In the time-course experiments described above, only a portion of HUVEC expressing HHV-6 early antigen progressed in the virus life-cycle to express late viral proteins. In permissive cells, the synthesis of viral proteins follows a cascade pattern with efficient synthesis of late gene products being dependent upon viral DNA replication. To determine whether this partial block in late gene expression occurred before or after HHV-6 DNA replication, infected HUVEC cultures were studied for their ability to support viral DNA synthesis. Serial dilutions of total DNA extracted from HHV-6-infected HUVEC, maintained in the presence or absence of PAA, or HHV-6-infected JJhan cells were examined by dot-blot analysis using a virus-specific probe. As illustrated in Fig. 4, at 4 days p.i. the amount of viral DNA present in HHV-6-infected HUVEC was comparable to that isolated from infected JJhan cells. Detection of viral DNA could not be attributed to input virus since treatment with PAA, a potent inhibitor of viral DNA replication, reduced the signal to a level observed in uninfected cells. In addition, a cell-free virus stock was used
HHV-6 infection of endothelial cells

Fig. 3. Time-course of HHV-6A infection. HUVEC monolayers were co-cultivated with HHV-6A-infected Jhan cells (A) or infected with HHV-6A cell-free supernatant (B). At the indicated times, HUVEC cultures were fixed and stained with MAbs p41 or 6A5G3, specific for HHV-6 early and late proteins, respectively. Four independent experiments were performed with similar results, and two representative experiments are shown. Values are presented as the mean ± SD of ten fields scored for each time point. (*) denotes the detection of no antigen-positive cells in ten fields. The total number of HUVEC present in infected cultures at each time point was determined and expressed as the percentage of mock-infected cells present at a given time-point (C). The actual number of cells (infected/mock-infected) is also given for each time-point.

Production of infectious virus

As outlined in the studies above, a limited number of endothelial cells can support HHV-6 late gene expression. To determine whether this population of cells can also generate and release infectious virus, culture supernatants were incubated with PBMC and assayed by indirect IF for the presence of HHV-6 antigens. Two separate experiments were performed, analysing culture fluids collected on days 6, 8, 10 and 12 p.i. The results from both experiments, at all time-points examined, were negative. As a positive control, PBMC were inoculated with cell-free virus stocks under identical conditions and assayed for early viral gene expression. These cultures were positive, proving that the PBMC cultures were susceptible to HHV-6 infection. The expression of early viral proteins in HHV-6-infected HUVEC cultures was also monitored in these experiments and ranged from 8.5–20%. This level of infection in permissive cells is sufficient for detecting the release of virus into the culture medium. Together, these results suggest that infectious virus was not released into the culture medium at detectable levels.

Persistent infection of HUVEC

To determine if HHV-6 can be maintained in HUVEC over an extended period of time, infected monolayers were
continuously passaged and monitored for HHV-6 antigen expression by indirect IF. In these studies, the initial level of infection was low in order to keep destruction of the monolayer to a minimum. The results from two independent experiments are shown in Table 1, with infection of HUVEC performed in triplicate under identical conditions. After the first passage the number of antigen-positive cells declined rapidly to less than 1%. This reduction is most notable in experiment 2 where the initial frequency of cells synthesizing early viral proteins was between 7 and 13%. Surprisingly, continued passage of HUVEC demonstrated the presence of a small population of endothelial cells expressing HHV-6 p41 antigen. The number of antigen-positive cells decreased slowly with passage, but positive cells were detected up to day 27 p.i. These results suggest that HHV-6 can establish a chronic infection in endothelial cells that can persist for up to 4 weeks.

When no antigen-positive cells were observed for two passages (passages 8 and 7 for experiments 1 and 2, respectively), HUVEC monolayers were overlaid with PBMC (experiment 1) or CBMC (experiment 2) for 24 h. PBMC/CBMC were removed from the monolayers and incubation continued for 10 days. This resulted in no recovery of virus in PBMC/CBMC cultures as determined by the lack of cytopathic effect and the absence of early viral gene expression. Treatment of the HUVEC monolayer with TPA or sodium butyrate prior to cocultivation had no effect on the outcome. Furthermore, HUVEC monolayers showed no evidence of HHV-6 gene expression after treatment. In contrast, HHV-6 was successfully isolated from monolayers that contained a small number of virus-infected cells. Table 2 shows the results from two independent experiments where the frequency of HHV-6 antigen-positive endothelial cells dropped to less than 1% after the first passage. At this time HUVEC were co-incubated with PBMC, and 10 days after co-cultivation positive PBMC were detected by indirect IF assay. Attempts to rescue virus from

Table 1. Chronic infection of HUVEC monolayers by HHV-6

HUVEC monolayers were infected with HHV-6A cell-free virus in triplicate (i–iii) and passaged, monitoring early viral gene expression by indirect IF. The results from two independent experiments are shown. The data represent the mean ± SD of ten fields scored.

<table>
<thead>
<tr>
<th>Passage no. (days p.i.)</th>
<th>HHV-6 antigen-positive cells (%)</th>
<th>Passage no. (days p.i.)</th>
<th>HHV-6 antigen-positive cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
<td></td>
<td>Experiment 2</td>
</tr>
<tr>
<td></td>
<td>(i)</td>
<td>(ii)</td>
<td>(iii)</td>
</tr>
<tr>
<td>0 (3)</td>
<td>3 ± 1.7</td>
<td>3 ± 1.7</td>
<td>2 ± 1.4</td>
</tr>
<tr>
<td>1 (8)</td>
<td>ND</td>
<td>ND</td>
<td>0.5 ± 0.9</td>
</tr>
<tr>
<td>2 (15)</td>
<td>0.5 ± 0.9</td>
<td>0.3 ± 0.9</td>
<td>0.1</td>
</tr>
<tr>
<td>3 (17)</td>
<td>0.4 ± 0.5</td>
<td>0.3 ± 0.9</td>
<td>0.1</td>
</tr>
<tr>
<td>4 (22)</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>5 (27)</td>
<td>0</td>
<td>0</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>6 (31)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7 (35)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8 (38)</td>
<td></td>
<td></td>
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</tbody>
</table>

*The value < 0.1% is given for no HHV-6 p41 antigen-positive cells observed in ten fields scored; however, on scanning the entire coverslip HHV-6-positive HUVEC were found.

ND, Not done.
To determine the effects of various cytokines and mediators on the susceptibility of endothelial cells to HHV-6 infection, early gene expression was monitored by indirect IF. The results from a representative study are shown in Table 3. When HUVEC were pretreated with either 0.5 or 2.5 µg/ml TNF-α, early gene expression was abolished. Yet even in the absence of early gene expression, multi-nucleated syncytia (a characteristic cytopathic effect of HHV-6 infection) were still observed. However, exposure of HUVEC to physiological concentrations of TNF-α resulted in no reduction of HHV-6 early proteins.

### Discussion

The purpose of these studies was to determine if HHV-6 could be transmitted from T-lymphocytes to the endothelium by cell-to-cell contact, establishing a model system for examining the dissemination of the virus from components of the blood through the vascular endothelium lining blood vessels to other tissues throughout the body. Many of the experiments in this report have employed HHV-6-infected lymphocytes as the source of infectious material, instead of cell-free virus, to more closely mimic the interactive cellular events occurring in vivo. Using indirect IF assays and dual immunohistochemical techniques, the results presented here demonstrate that endothelial cells are susceptible to HHV-6 infection. Both HHV-6 early and late viral gene products were detected in HUVEC monolayers exposed to isolate U1102 or R255, representing HHV-6 strains A and B, respectively (Fig. 1). Such an interactive exchange between circulating blood components and the endothelium has also been observed for human cytomegalovirus (HCMV), another betaherpesvirus (Waldman et al., 1995). In this case transfer of virus was from endothelial cells, a natural host for HCMV in vivo, to monocytes.

Although HHV-6 is capable of infecting HUVEC, only a small percentage of endothelial cells expressing early antigens progressed in the viral cascade to produce late proteins (Fig. 3 A, B) and attempts to detect the release of infectious progeny in the culture supernatant were unsuccessful. In permissive cell lines, the ratio of virus particle to infectivity is low, approximately 1000 to 1 (Skiraki et al., 1991). However, adjusting for this fact and the lower frequency of infection in HUVEC monolayers, the sensitivity of the assay is still sufficient to achieve detection of released virus. These findings suggest that HHV-6 infection of HUVEC is only semi-permissive in nature.

HHV-6 infection of other non-immune cells, including fibroblasts, epithelial cells and astrocytes, also appears to be somewhat restricted, resulting in virus titres that are 100- to 1000-fold lower than those produced in CD4⁺ T-lymphocytes, the natural host for HHV-6 (Luka et al., 1990; Simmons et al., 1992; He et al., 1996). The biological basis for efficient replication of HHV-6 only in lymphoid cells is uncertain. It is interesting to note that while HCMV can productively infect human brain capillary endothelial cells (Lathey et al., 1990; Poland et al., 1990), the ability of the virus to replicate in vivo is compromised.

### Table 2. Recovery of HHV-6 from infected endothelial cells

HUVEC monolayers were mock infected or infected with HHV-6A cell-free virus and passaged once, monitoring early viral gene expression. The number of HHV-6 antigen-positive HUVEC present after the first passage is indicated. HUVEC monolayers were overlaid with PBMC. The number of HHV-6-positive PBMC was determined 10 days after co-cultivation by IF. The results from two independent experiments are shown. The data represent the mean ± SD of ten fields scored.

<table>
<thead>
<tr>
<th>Expt</th>
<th>HHV-6 antigen-positive HUVEC (%)</th>
<th>HHV-6 antigen-positive PBMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HJV-6A infected</td>
<td>0.4 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Mock infected</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>HHV-6A infected</td>
<td>0.7 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>Mock infected</td>
<td>0</td>
</tr>
</tbody>
</table>

### Table 3. Effect of various cytokines on HHV-6 early gene expression in HUVEC monolayers

Four independent experiments were performed. The results from a representative experiment are shown. The data are the mean number of cells ± SD in ten fields. * Significant at $P < 0.01$; ** significant at $P < 0.05$. Statistical analysis was performed using a Student’s t-test.

<table>
<thead>
<tr>
<th>Pretreatment of HUVEC</th>
<th>HHV-6 antigen-positive cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>12.5 ± 7.3</td>
</tr>
<tr>
<td>LPS (5 µg/ml)</td>
<td>12.8 ± 6.4</td>
</tr>
<tr>
<td>TNF-α (0.5 µg/ml)</td>
<td>0*</td>
</tr>
<tr>
<td>TNF-α (2.5 µg/ml)</td>
<td>0*</td>
</tr>
<tr>
<td>IL-1α (125 units/ml)</td>
<td>8.0 ± 8.2</td>
</tr>
<tr>
<td>IFN-α (10000 units/ml)</td>
<td>8.3 ± 11.0</td>
</tr>
<tr>
<td>IFN-α (50000 units/ml)</td>
<td>7.6 ± 3.9**</td>
</tr>
<tr>
<td>IFN-γ (2000 units/ml)</td>
<td>8.1 ± 5.0</td>
</tr>
<tr>
<td>IFN-γ (10000 units/ml)</td>
<td>8.8 ± 5.5**</td>
</tr>
</tbody>
</table>

mock-infected HUVEC, passaged once prior to co-cultivation with PBMC, were negative. The ability to recover virus from infected HUVEC indicates that endothelial cells can serve as a reservoir for HHV-6.

IFN-α, IFN-γ and TNF-α inhibit HHV-6 early gene expression in HUVEC

Several studies have shown that infection of PBMC with HHV-6 can induce alterations in the production of cytokines (Kikuta et al., 1990; Flamand et al., 1991; Gosselin et al., 1992). To determine the effects of various cytokines and mediators on
efficiently in HUVEC is limited (Smiley et al., 1988; Wu et al., 1994). These studies emphasize the biochemical and phenotypic heterogeneity that exists between microvascular and large-vessel endothelial cells (Zetter, 1988).

The number of infected HUVEC identified in the time-course experiments (Fig. 3 A, B) may in fact be an underestimation due to loss of cells from the monolayer during the course of HHV-6 infection (Fig. 3 C). Whether these unattached cells represent HHV-6-infected endothelial cells, uninfected HUVEC or a mixed population remains to be elucidated. Non-specific staining of non-viable cells does not allow this question to be addressed with indirect IF techniques. However, two plausible explanations for the loss of HUVEC come to mind. Firstly, HHV-6 infection could result in the release of a soluble factor that is toxic to HUVEC and thus lead to cell death. HHV-6 has been documented to induce the secretion of various cytokines (Kikut et al., 1990; Flamand et al., 1991; Gosselin et al., 1992). However, in this study incubation of HUVEC cultures with TNF-α, IL-1, INF-α or INF-γ did not lead to appreciable cell loss (unpublished results). Therefore, we favour a second explanation which involves HHV-6 as a direct cause of cell lysis and destruction. The relationship between HHV-6 and its host has been well documented for T-lymphocytes. Here, infection by HHV-6 causes the development of large refractile cells that die within 10–12 days (Salahuddin et al., 1986). In a second study, a comparison of the number of cells present in HHV-6-infected versus mock-infected CBMC 5 days after infection revealed a 20% decline in infected cultures, an effect that was consistent in numerous experiments (Black et al., 1991). In addition, HHV-6 can suppress cell proliferation and IL-2 production (Horvat et al., 1993; Flamand et al., 1995), as well as shut off host-cell DNA replication (DiLuca et al., 1990). Further studies must be undertaken to determine if HHV-6 has a similar effect on its endothelial cell host.

The present data suggest that HHV-6 does not replicate productively in HUVEC. However, the virus was maintained in continually passaged HUVEC for almost a month (Table 1) and HHV-6 was recovered from these monolayers by co-cultivation with permissive PBMC. These findings indicate that endothelial cells can serve as a repository for chronic HHV-6 infection. Our inability to rescue the virus from HUVEC monolayers that were HHV-6 antigen negative for two passages is consistent with the idea that while the virus can persist in these cells, the endothelium is not a site for viral latency. However, it is possible that our attempts to recover HHV-6 have met with failure due to culture conditions. Kondo et al. (1991) have reported that HHV-6 can be reactivated from human monocytes/macrophages in vitro only after pretreatment with TPA for 5 days. Our HUVEC cultures were pretreated with TPA, but only for 24 h due to the sensitive nature of the cells. Katsafanas et al. (1996) have described the reactivation of HHV-6 from latently infected PBMC in vitro, a process that is dependent on active infection with human herpesvirus-7 (HHV-7). They concluded that HHV-7 provides a trans-acting function necessary for the rescue of HHV-6. Studies are now under way in our laboratory to test whether such factors will influence HHV-6 recovery from HUVEC.

Defence mechanisms designed to protect the host against invading viruses activate a number of cell-mediated immune strategies, including the release of cytokines. These soluble regulatory elements, secreted primarily by monocytes/macrophages, serve an important function in virus clearance from the infected site (Ramsay et al., 1993). HHV-6 infection of PBMC elicits the synthesis of TNF-α, IL-1 and IFN-γ (Kikut et al., 1990; Flamand et al., 1991; Gosselin et al., 1992), mediators that exert a profound influence on the structure and function of the endothelium (Pober, 1992; Mantovani et al., 1992). Using our model system, we examined the effect of these cytokines on the susceptibility of endothelial cells to HHV-6 infection in vitro, to help define their potential role in preventing the spread of virus in vivo. Exposure of endothelial cells to TNF-α prior to viral infection induced the most pronounced response – complete inhibition of early antigen expression (Table 3). However, such an effect occurred only at concentrations 1000-fold greater than physiological levels. The antiviral effects of TNF-α are well established for alphaherpesviruses. TNF-α selectively kills herpes simplex virus type 1- and type 2-infected cells by accelerating cell lysis, leaving uninfected cells intact (Wong & Goeddel, 1986, Koff & Fann, 1986; Ito & O’Malley, 1987). This antiviral effect of TNF-α is synergistically enhanced by the presence of IFN-γ, inhibiting replication of the virus more efficiently than can be observed with either molecule alone (Wong & Goeddel, 1986; Feduchi et al., 1987). The combined antiviral activity of TNF-α and IFN-γ has also been reported for betaherpesviruses. While high concentrations of TNF-α or IFN-γ alone were necessary to invoke any antiviral activity, when tested in combination the effective concentrations of these two mediators were reduced to physiological levels (Lucin et al., 1994). Whether combined therapy would reduce the levels of TNF-α necessary to inhibit HHV-6 synthesis is currently being tested.

With regard to pathogenicity and spread of the virus, the present studies demonstrate two important findings: (a) HUVEC can serve as a reservoir for HHV-6 in vitro, and (b) HHV-6 infection of HUVEC leads to cell loss. Thus the susceptibility of the endothelium to HHV-6 infection may help explain the widespread nature of the virus in immunocompromised patients.

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