Human Cytomegalovirus Isolates Differ in Multiplication and Induction of IL-8 in Epithelial Cells

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Abstract
Epithelial cells are major targets of permissive infection by human cytomegalovirus (HCMV) in vivo during acute infection, but little is known about the viral factors that influence the permissiveness of epithelial cells for HCMV. The aim of the present study was to investigate the interleukin-8 (IL-8)-inducing capacities of HCMV clinical isolates of different origins by comparison of the replication in A549 epithelial cells. The IL-8-inducing capacities of the HCMV isolates differed in A549 cells; the IE antigen expression and the virus yield proved to be dependent on the amount of IL-8 produced. Exogenous IL-8 increased the number of IE antigen-producing cells in a dose-dependent manner. Our observations indicate that certain HCMV strains induce high levels of IL-8 and augment their replication in the epithelial cells, and suggest that in vivo the replication of the others can be facilitated if IL-8 is provided by different sources.

ABBREVIATIONS
ELISA: Enzyme-Linked Immunosorbent Assay; FCS: Fetal Calf Serum; HCMV: Human Cytomegalovirus; IE: Intermediate-early; IL-8: Interleukin-8; MEM: Minimum Essential Medium; rhIL-8: Recombinant Human IL-8

INTRODUCTION
Human cytomegalovirus (HCMV), also known as human herpesvirus 5, belongs in the subfamily Betaherpesvirus of the family Herpesviridae. HCMV displays a broad host cell range and infects many different cell types [1]. Epithelial cells are major targets of permissive infection by HCMV and play a role in the spread of the virus in infected tissues during acute infection. Little is known, however about the viral factors that influence the permissiveness of epithelial cells for HCMV [2]. Potential differences between HCMV strains were not taken into consideration.

We recently observed that syncytiotrophoblasts infected with HCMV isolates induced different amounts of interleukin-8 (IL-8), and that the immediate-early (IE) viral gene expression was positively influenced in an IL-8 dose-dependent manner [3]. It is known that HCMV replication in human fibroblast cell line is enhanced by IL-8 [4].

The aim of the present study was to investigate the IL-8-inducing capacities of clinical isolates of HCMV by comparison of the replication in another epithelial cell, the human A549 lung epithelial cell. To resemble a natural infection, a low MOI was used. The interrelationships between the amount of IL-8, the percentage of nuclei positive for HCMV IE antigen and the replication of the virus were examined. The effect of exogenous IL-8 on the IE antigen expressions of HCMV isolates with different IL-8-inducing abilities was investigated.

MATERIALS AND METHODS
Cells
Cells of the human A549 lung carcinoma cell line were cultivated in Eagle’s minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) and antibiotics. The cells were grown on glass coverslips in 24-well plates containing 2x10^5 cells/well for cytokine production, immunofluorescence and virus replication studies.

Viruses
HCMV clinical isolates of different origins (128V, 20, E33, E5, S and 74) and laboratory-adapted strain Towne ATCC #VR-977 (American Type Culture Collection) were used. HCMV strains 128V and E33 were isolated in our laboratory from the urine of congenitally infected neonates, and E5 from the urine of an adult. HCMV S was isolated by Dr. J. Schirm (Regional Health Laboratory, Groningen, The Netherlands) from the urine of a neonate. Isolates 20 and 74 originated from the blood of renal transplant recipients and were kindly provided by Professor C. Bruggeman (University Hospital, Maastricht, The Netherlands). The clinical isolates were passaged < 5 times. The stocks of HCMV strains were propagated in confluent MRC-5 cells grown in RPMI medium supplemented with 10% FCS and antibiotics.
The infectivity titeres were determined by plaque assay, with inoculation of confluent MRC-5 cultures in 24-well plates.

**Determination of cytokine production**

In order to determine the production of IL-8 by HCMV-infected A549 cells, the cell cultures were infected with HCMV strains of different origins or the laboratory-adapted strain at an MOI of 0.1. The infected cultures were centrifuged at 750xg for 60 min in a Heraeus Megafuge 1.0 (Osterode, Germany) at room temperature and then incubated for 2 hrs at 37°C. The unabsorbed virus was removed and the cells were washed 5 times with serum-free MEM. After washing, the cells were overlayed with MEM supplemented with 1% FCS and antibiotics. Supernatants of virus-infected and mock-infected cultures were collected at different time intervals after infection and assayed for IL-8 with an ELISA kit, following the manufacturer’s protocol. The ELISA kit was purchased from Biosource Europe SA, Nivelles, Belgium. As concerns the sensitivity of the IL-8 assay, the detectable dose of human IL-8 (hIL-8) was 5 pg/ml. The results on the variability in IL-8 secretion among cultures from three independent experiments are presented as the means ± standard deviation (SD).

**Immunofluorescence assay**

To monitor the efficiency of infection at 48 h p.i., immunofluorescence assays were used to detect of HCMV IE antigen-producing cells. The cultures were washed twice with cold phosphate-buffered saline and fixed with a cold acetone:ethanol (1:1) mixture for 20 min at -20°C. The fixed cells were stored at -20°C until immunofluorescence assays were performed [4]. The nuclei of the cells were stained with 4’,6-diamidino-2-phenylindole (DAPI), and the HCMV-infected cells were identified by IE antigen staining, using monoclonal antibody (MAB810) (Chemicon International Inc., Temecula, CA, USA) and fluoresce in isothiocyanate-conjugated rabbit anti-mouse IgG (Sigma, Budapest, Hungary).

The mean percentage ± SD of IE antigen-positive cells per 30 microscopic fields was calculated. Total cell numbers were determined via the numbers of DAPI-stained nuclei.

**Assay for virus replication**

Replication of HCMV strains 128V, E5 and Towne was determined by exposure of cells to viruses in a manner identical to that used for IL-8 induction. After exposure, culture supernatants were collected at zero-time point and thereafter daily, and made cell-free by centrifugation. Cell-associated virus was collected by three freeze-thaw cycles of infected cells in medium. Cell-free and cell-associated virus-containing samples collected at the same time were pooled. The virus titers were determined by plaque assay with inoculation of confluent MRC-5 cultures in 24-well plates. All assays were performed in triplicate. The virus reported titers are averages ± SD from three independent experiments.

**Determination of the effect of exogenous IL-8 on IE antigen expression**

A549 cells were incubated with different concentrations of recombinant human IL-8 (rhIL-8) (R&D Systems Europe Ltd.) for 1 h at 37 ºC. After removal of the medium, the cells were exposed to viruses in a manner identical to that used for IL-8 induction. The infected cells were overlayed with MEM with 1% FCS and antibiotics in the presence or absence of the various concentrations of IL-8. After 48-h incubation, immunofluorescence assays were performed for the detection of IE antigen-producing cells [5].

**RESULTS AND DISCUSSION**

IL-8 was detected in the supernatant of the mock and virus-infected A549 cultures at 6 h post infection and the amounts of IL-8 measured increased in a time-dependent manner, but did not rise further after 48 hrs. (data not shown).

Analyses of the IL-8 levels in the supernatants of the A549 cultures at 48 h post infection revealed that HCMV 128V was the most potent IL-8 inducer, followed in sequence by E5, 20, 74, S, E33 and Towne strain (Table 1).

The percentages of cells positive for IE antigen in the A549 cultures infected with the different HCMV strains seemed to be dependent on the amount of IL-8 produced (Table 1). A very low incidence of infection was seen for the laboratory-adapted strain Towne.

Three HCMV strains with different IL-8-inducing capacities were chosen to investigate the interrelationship between the level of IL-8 secreted and the replication of the virus in A549 cells. The single-step growth curves of the HCMV isolates at an MOI of 0.1 in A549 cells demonstrated the production of infectious virus. There was a 4 log difference between the highest titers of HCMV 128V and E5 (Figure 1). In the cell culture infected with the laboratory-adapted Towne strain, no infectious virus was detected. The greater the amount of IL-8 produced in the HCMV-infected A549 culture, the higher the yield of infectious virus.

The results support our earlier suggestion that interstrain differences in multiplication of HCMV in epithelial cell depend on the IL-8-inducing capacity and influence the outcome of HCMV infection [3].

Exogenous IL-8 increased the number of IE antigen-producing cells at concentrations of from 0.1 to 10 pg of IL-8 per ml, in a

<table>
<thead>
<tr>
<th>HCMV</th>
<th>IL-8 (pg/ml)</th>
<th>IE antigen-positive nuclei (%)</th>
</tr>
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<tbody>
<tr>
<td>-</td>
<td>135±38</td>
<td>-</td>
</tr>
<tr>
<td>128V</td>
<td>5009±835</td>
<td>3.5 ± 0.7</td>
</tr>
<tr>
<td>E33</td>
<td>435±48</td>
<td>1.4 ± 0.6</td>
</tr>
<tr>
<td>E5</td>
<td>2215±305</td>
<td>1.8 ± 0.7</td>
</tr>
<tr>
<td>20</td>
<td>687±172</td>
<td>ND</td>
</tr>
<tr>
<td>S</td>
<td>469±59</td>
<td>ND</td>
</tr>
<tr>
<td>74</td>
<td>545±64</td>
<td>ND</td>
</tr>
<tr>
<td>Towne</td>
<td>156±25</td>
<td>0.5 ± 0.1</td>
</tr>
</tbody>
</table>

The values are means ± SD

**Abbreviations:** SD: Standard Deviation; ND: Not done; IL-8: Interleukin-8; IE: Intermediate-Early; HCMV: Human Cytomegalovirus
dose-dependent manner (Figure 2).

Exogenous IL-8 has been demonstrated to upregulate the replication of laboratory-adapted HCMV strains in various cells [4,6,7]. Our results indicate that exogenous IL-8 increases the number of IE antigen-producing cells, in a dose-dependent manner, in A549 cells infected with HCMV strains with different IL-8-inducing abilities. The presence of this cytokine in the microenvironment may play a regulatory role, potentially promoting the progression of HCMV infection.

Our findings suggest that certain HCMV strains induce a high level of IL-8 in epithelial cells, which in turn enhances productive HCMV infection in these cells. Other HCMV strains can replicate in epithelial cells if IL-8 is provided by cofecting agents: viruses or bacteria or other sources. The potential of various bacteria to stimulate IL-8 production in epithelial cells varies [8,9].

It has become clear in recent years that many HCMV genes differ strikingly in sequence between strains, with at least 25 of the 165 genes present in wild-type virus strains being hypervariable [10,11]. The anticipated association between pathogenic properties and genotypes is a subject of importance, but has yet to find conclusive experimental support. We focus on several hypervariable genes whose genotypic structures are well characterized and which encode products with potentially relevant functions [12]. The key target is UL146, which encodes a potent CXC chemokine similar to IL-8 [13]. HCMV strains (128V, 20, E33, E5, S and 74) for which genotype data were obtained [12] and included in the experiments of our present paper, four UL146 genotypes (1, 7, 9 and 12) were apparent (unpublished data).

The number of strains whose ability to induce IL-8 has been examined is limited, and only one strain (128V) induces high levels. A correlation with UL146 remains possible, since no other strain has the same genotype (9) as strain 128V. The analysis of additional strains is required, especially those able to induce high levels of IL-8 or those known to have a genotype 9 UL146 gene. Further investigation will help to determine whether particular genotypes of UL146 (and other genes) are associated with IL-8 induction in physiologically relevant syncytiotrophoblast cells and other epithelial cells.

**CONCLUSION**

This study using HCMV clinical isolates contribute to our understanding of the roles of viral factors in the multiplication of HCMV in epithelial cells. The knowledge of the molecular basis of the interactions between HCMV isolates and epithelial cells may yield novel strategies with which to prevent the progression of the virus. IL-8 may be an important new target for the control of HCMV replication.

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