In Vitro Generation of Human Cytomegalovirus pp65 Antigenemia, Viremia, and LeukoDNAemia

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Abstract

Immunocompromised patients with disseminated human cytomegalovirus (HCMV) infection have circulating PMN carrying HCMV pp65 (antigenemia), infectious virus (viremia), and viral DNA (leukoDNAemia). Because HCMV does not fully replicate in PMN, it is generally hypothesized that virions and viral materials are taken up by phagocytosis from fully permissive HCMV–infected endothelial cells. However, no experimental evidence has ever been provided for these PMN–endothelium interactions. PMN from 11 donors were cocultured with endothelial cells infected with an endothelium-adapted HCMV strain and with human fibroblasts infected with low-passaged clinical and laboratory-adapted HCMV strains. pp65-positive PMN were detected after coculture with both HCMV-infected endothelial and fibroblast cells, provided that wild and not laboratory-adapted strains were used. In addition, cocultured PMN carried infectious virus as demonstrated by virus isolation and presence of complete virus particles by electron microscopy. Moreover, high levels of viral DNA were consistently detected by quantitative PCR in cocultured PMN. Thus, we have generated in vitro the three most important viral parameters detected in patients with disseminated HCMV infection (antigenemia, viremia, and leukoDNAemia). The failure of laboratory-adapted HCMV strain to induce this phenomenon demonstrates that important modifications have occurred in attenuated viral strains affecting basic biological functions. (J. Clin. Invest. 1998. 101:2686–2692.) Key words: endothelial cells • fibroblasts • cocultivation • polymorphonuclear leukocytes • immunocompromised patients

Introduction

One of the most rapid approaches to the diagnosis and monitoring of systemic human cytomegalovirus (HCMV) infection is the detection of HCMV antigens in nuclei of peripheral blood leukocytes and, in particular, of PMN. This assay is known as the antigenemia test (1). The major HCMV antigen detected in this assay is the 65-kD lower matrix phosphoprotein or pp65 (ppUL83; 2). In addition to pp65, PMN can carry infectious HCMV particles that can be recovered after cocultivation with human embryonic lung fibroblast (HELF) cells (3). Although early replicative events do seem to occur in PMN (4, 5), it is currently recognized that pp65 and infectious virus are mostly uptaken by phagocytosis. From a pathogenic standpoint, it has been speculated that HCMV-infected leukocytes can contribute to spreading of HCMV infection by infecting vascular endothelial cells (EC; 6, 7). Conversely, HCMV-infected EC are thought to be the most likely source of both infectious virus and pp65 for PMN, although no evidence for such a phenomenon has ever been reported either in vivo or in vitro. In this report, we demonstrate that PMN can uptake HCMV pp65 and infectious virus in vitro after cocultivation with either virus-infected EC or HELF, provided that wild and not laboratory-adapted HCMV strains are used.

Methods

Cell cultures and virus strains. Human umbilical vein endothelial cells (HUVEC) were obtained by trypsin treatment of umbilical cord veins, propagated as reported (8), and used at passages 5–10. Culture medium for HUVEC was M199 supplemented with 20% FCS, endothelial cell growth factor (Sigma Chemical Co., St. Louis, MO), and 5 U/ml sodium heparin. In addition, a HELF cell strain originally developed in our laboratory was used at passages 20–30. Culture medium for HELF was Earle’s minimum essential medium supplemented with 10% FCS in the growth medium and 2% FCS in the maintenance medium.  

An HCMV strain (VR#6110) recovered from blood of an AIDS patient was adapted to growth in HUVEC. After three initial passages in HELF cultures, VR#6110 underwent 28 passages in HUVEC (VR#6110/28) before use. In addition, seven clinical HCMV isolates, including VR#6110, were propagated in HELF cultures and used at passages 2–20. The seven HCMV strains were isolated from urine, amniotic fluid, human milk, lung biopsy, cervix, and buffy coat (two strains), respectively. HCMV laboratory strains AD169, Davis, and Towne (American Type Culture Collection, Rockville, MD) were propagated in our laboratory for several years. Cells and viral strains were routinely checked for mycoplasma contamination by Hoechst staining (9).

Isolation of PMN. Human PMN were obtained from seven HCMV-seropositive and four HCMV-seronegative healthy donors. HCMV serostatus was determined by using a previously described ELISA assay (10). 1 ml of 6% dextran solution (molecular weight 70,000) in saline was added to 5.0 ml of heparinized blood. After incubation at 37°C for 30 min, the supernatant was dispensed onto Ficoll-Hypaque and centrifuged at 600 g for 35 min. Contaminating red blood cells were removed by hypotonic lysis (1 ml 0.8% NH4Cl for 2 min). The purity of harvested PMN was >95%, as assessed by May Grünwald-Giemsa as well as by chloro-acetate esterase staining. Cell
viability was determined by trypan blue exclusion and was consistently > 95%.

Cocultivation of PMN and HCMV-infected HUVEC or HELF. HCMV-infected HUVEC or HELF monolayers showing > 80% cytopathic effect were trypsinized. Infected cells were then mixed with uninfected HUVEC or HELF cells at 1:2 ratio and plated in 24-well cell culture plates (10<sup>5</sup> cells/well). After 72 h, monolayers were washed and inoculated with 4 × 10<sup>8</sup> PMN. Cocultivation was continued for 24 h at 37°C unless otherwise indicated.

**pp65 detection in PMN after cocultivation.** PMN were recovered from HCMV-infected monolayers by gentle pipetting and aspiration of medium. Supernatants were centrifuged at 1,200 rpm for 5 min, and cell pellets were washed twice, counted, and suspended at a concentration of 2 × 10<sup>5</sup> cells/ml. 100 µl (2 × 10<sup>5</sup> cells) were then used for cytospin preparations that were fixed and stained using a previously reported procedure (2, 11). The number of pp65-positive PMN was counted and recorded. pp65-positive HUVEC or HELF cells were easily distinguished from pp65-positive PMN by size and by the predominant cytoplasmic staining, in contrast to the exclusive PMN nuclear staining. In addition, detection of pp65-positive PMN was also performed in cytopsin preparations of migrated or FACS<sup>®</sup>-purified PMN, as described below.

In some experiments, PMN suspensions were incubated with HCMV-infected HUVEC for 1 h at 37°C. Supernatant containing unbound cells was then harvested and processed as described below. The EC monolayer was carefully washed to remove any residual, unbound PMN, refed with fresh medium, and cocultivation continued for 24 h. At the end of the cocultivation time, supernatants containing de-adhered HCMV were harvested and centrifuged. The adhering PMN were recovered by trypsin treatment of HCMV-infected HUVEC monolayers. Both cell fractions (adhering and nonadhering collected at 1- and 24-h coculture) were then washed twice, fixed, permeabilized, and stained for pp65 with specific mAb after a reported procedure for pp65 antigenemia determination (11). In parallel preparations, an irrelevant isotype-matched mAb was used. Samples were then analyzed on a FACSScan<sup>®</sup> (Becton Dickinson, San Jose, CA) flow cytometer. The instrument setting was preliminarily carried out using isolated PMN and HUVEC to create gated cytograms corresponding to the physical parameters (forward scatter and side scatter) of each cell subset. Staining of the cocultured cells using a myeloid specific marker (anti-CD 18 mAb TS.18, generously provided by T.A. Springer, Harvard University, Cambridge, MA) confirmed that > 95% of cells in the PMN gate and < 5% of cells in the HUVEC gate were of myeloid lineage.

**PMN-mediated HCMV transmission and viral DNA detection in cocultured PMN.** After cocultivation, to separate PMN from infected cells, 5 × 10<sup>3</sup>-10<sup>5</sup> PMN in 250 µl RPMI 1640 (Flow Laboratories, Irvine, Scotland) supplemented with 1% FCS (GIBCO BRL, Life Technologies, Paisley, Scotland) were placed in the upper compartment of each cell culture insert (5 µm pore size, 6.5 mm diameter Transwell<sup>®</sup>; COSTAR, Cambridge, MA) and incubated for 3 h at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. The bottom compartment contained 10<sup>6</sup> M FMLP (Sigma Chemical Co., St. Louis, MO) in 500 µl RPMI 1640 plus 1% FCS (12). After incubation, the chambers were shaken to dislodge PMN from the lower surface of the inserts. PMN were then collected, extensively washed, and then inoculated (2 × 10<sup>6</sup> cells) onto HELF or HUVEC monolayers grown in shell vials. Early identification of infectious virus was then accomplished 16–24 h post infection (p.i.) by using the indirect immunofluorescence technique and a mAb to the HCMV major immediate early protein p72 (3). p72-positive nuclei were then counted and the number recorded. In parallel, HCMV DNA was quantitated in 10<sup>6</sup> PMN aliquots recovered after migration according to a reported PCR procedure using a primer pair relevant to exon 4 of the major immediate early gene (primer N1, nt 941–959; N2, nt 1431–1412) (13). In some experiments, PMN aliquots were treated with deoxyribonuclease (Boehringer Mannheim GmbH, Mannheim, Germany) for 30 min before DNA extraction. In addition, 2 × 10<sup>6</sup> aliquots of migrated PMN were used for cytopsin preparations for pp65 detection, as described above. Purity of migrated PMN was assessed by the May-Grünwald staining and was consistently > 95%. Similarly, cell viability was > 95%.

**PMN purification by FACS<sup>®</sup>.** In some experiments, the migrated fraction of PMN was further purified by FACS<sup>®</sup> after staining of the cells for CD 66b (Immunotech, Instrumentation Laboratory, Milan, Italy). Cell sorting was performed with a FACStar<sup>®</sup> PLUS (Becton Dickinson, Mountain View, CA). Laser output was 240 mW at 488 nm. Sorting was carried out according to standard FACStar<sup>®</sup> specifications with a three-drop deflection criterion using a 70-µm nozzle. The sorted PMN fraction was then used for quantification of pp65-positive cells, infectious virus, and viral DNA. The same parameters were determined on aliquots of migrated PMN before FACS<sup>®</sup> purification.

**Blocking experiments.** PMN were first cultured into trans-well culture inserts of 0.4-µm pore membrane (Becton Dickinson, Bedford, MA) that prevented PMN contact with the underlying cell monolayer. In addition, PMN were pretreated for 30 min with 6 µg/ml of an mAb directed against the common beta chain (CD18) of lymphocyte function-associated antigen (LFA-1; DAKO, Glostrup, Denmark). Furthermore, HCMV-infected HUVEC or HELF monolayers were preincubated for 30 min with 15.2 µg/ml of an anti-intracellular adhesion molecule 1 (ICAM-1) mAb (DAKO). Cocultures were then carried out in the presence of either mAb or of an irrelevant, isotype-matched control mAb.

**Electron microscopy (EM).** In EM studies, cocultured PMN were fixed with Karnovsky’s fixative, fixed afterwards with 1.5% OsO<sub>4</sub> in 0.2 M cacodylate buffer, pH 7.3, dehydrated, and embedded in Epon-ARaldite resin either before or after purification. Ultrathin sections were stained with lead citrate and uranyl acetate, and examined with a Zeiss 902 electron microscope (Oberkochen, Germany).

## Results

**HCMV pp65 detection in cocultured PMN.** In initial experiments, PMN obtained from one seronegative and one seropositive subject were either incubated with AD169 cell-free suspension or cocultured with AD169-infected HELF cells. pp65-specific staining was never observed in the nucleus of PMN.

However, when PMN from the same two subjects were cocultured with HUVEC infected with VR6110/28<sub>G</sub>, pp65 was readily detected in the nucleus of PMN from both individuals with the same staining pattern observed ex vivo in patients

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Figure 1. Immunofluorescent staining of HCMV pp65 in the nucleus of PMN recovered after a 24-h coculture with HUVEC infected with VR6110/28<sub>G</sub>.
with pp65 antigenemia (Fig. 1). To assess whether VR#6110/28E–infected HUVEC could release soluble factor(s) responsible for (mediating) pp65 uptake by PMN, cocultivation experiments (Table I) were performed using clarified supernatants collected from VR#6110/28E–infected (and mock-infected) HUVEC cultures as conditioned (and control) medium. Cocultivation of PMN and AD169-infected HELF in the presence of conditioned medium did not result in the detection of any pp65-positive PMN, whereas pp65-positive PMN could be detected after cocultivation with HELF cultures infected with cell-free VR#6110/28E both in the presence of conditioned and control medium (Table I). These results also demonstrated that fibroblasts infected with VR#6110/28E could efficiently induce pp65 uptake by PMN.

This raised the question of whether the phenomenon was strain-specific (VR#6110/28E) or extended to any clinical strain. Results reported in Table I show that pp65-positive PMN could be detected after cocultivation with HELF infected with any of seven clinical HCMV isolates at passage 2–20 (including an early passage of VR6110), but not with HELF infected with any of the laboratory-adapted strains (AD169, Towne, Davis).

<table>
<thead>
<tr>
<th>PMN coculture condition</th>
<th>pp65-positive PMN</th>
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<tbody>
<tr>
<td>VR#6110/28E–infected HUVEC present</td>
<td>AD169-infected HELF + conditioned medium* absent</td>
</tr>
<tr>
<td>AD169-infected HELF + control medium*</td>
<td>VR#6110/28E–infected HELF + control medium present</td>
</tr>
<tr>
<td>VR#6110/28E–infected HELF + conditioned medium present</td>
<td></td>
</tr>
<tr>
<td>VR#610/4r (buffy coat)–infected HELF</td>
<td>VR#610/4r (buffy coat)–infected HELF present</td>
</tr>
<tr>
<td>VR#347/2f (urine)–infected HELF</td>
<td>VR#347/2f (urine)–infected HELF present</td>
</tr>
<tr>
<td>VR#348/2f (amniotic fluid)–infected HELF</td>
<td>VR#348/3f (human milk)–infected HELF present</td>
</tr>
<tr>
<td>VR#340/3f (human milk)–infected HELF</td>
<td>VR#343/3f (lung biopsy)–infected HELF present</td>
</tr>
<tr>
<td>VR#1814/20f (cervix)–infected HELF</td>
<td>VR#1824/20f (cervix)–infected HELF present</td>
</tr>
<tr>
<td>Towne-infected HELF</td>
<td>VR#1824/20f (cervix)–infected HELF present</td>
</tr>
<tr>
<td>Davis-infected HELF</td>
<td>VR#1824/20f (cervix)–infected HELF present</td>
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</table>

*Clarified medium collected from VR#6110/28E–infected (and mock-infected) HUVEC cell cultures.

Table I. Relationship of Type of Cell Culture and HCMV Strains in Generating pp65-positive PMN

Kinetics and frequency of pp65 uptake by PMN after cocultivation. PMN were examined for pp65 staining after a 1-, 6-, 24-, 48-, 72-, and 96-h cocultivation with either HUVEC or HELF VR#6110/28E–infected monolayers. In a small number of PMN (1–2% of the peak value), pp65 was detected as early as after 1 h (Fig. 2A). Number of pp65-positive cells increased in time reaching a peak after a 48-h coculture.

As for PMN nuclear morphology, > 90% pp65-positive PMN recovered after a 24-h coculture had < 4 lobes, whereas at later times frequency of pp65-positive PMN exhibiting a multilobed nucleus (5–10 lobes) slightly increased. Based on this observation and the additional finding that PMN recovered after a 48-h coculture showed reduced migration capacity (see below), a 24-h cocultivation was chosen as the optimal time for standard experiments.

Frequency of pp65-positive PMN varied from 0.2 to 5% and appeared directly related to the degree of infection of the cell monolayer. In fact, when PMN were cocultured for 24 h with HELF cells infected for 96 h with log_{10} dilutions of a low-passage cell-free HCMV strain (VR#6347/2f), a progressive increase of pp65-positive PMN was observed in parallel with increasing amounts of input virus (Fig. 2B).
To investigate pp65 uptake in relation to the time-course of HCMV infection, HELF monolayers were infected with $10^{-1}$ dilution of VR6347/2p. PMN were then added to monolayers 1, 24, 48, 72, 96, and 120 h p.i., and cocultivated for 3 h. As shown in Fig. 2 C, pp65-positive PMN were not detected until 72 h, and then their relative number increased up to 120 h p.i.

PMN-mediated transmission of infectious virus and detection of viral DNA in cocultured PMN. The unequivocal demonstration that PMN cocultured with HCMV-infected cells did not only express pp65, but could also transmit infectious virus, required removal of all HCMV-infected cells (either HUVEC or HELF) present in the PMN suspension recovered after cocultivation. This was achieved by taking advantage of the capacity of PMN to migrate through natural and artificial barriers in response to chemoattractants. Control experiments indicated that neither HUVEC nor HELF (infected or mock infected) were able to migrate in response to FMLP. On the other hand, 30–50% of PMN recovered after a 24-h coculture with HCMV-infected or mock-infected HUVEC or HELF monolayers, consistently migrated in response to FMLP, whereas PMN recovered after 48- and 72-h cocultures showed decreasing migrating capacity from 20 to $\sim 1\%$, respectively. In addition, PMN positive for pp65 appeared to migrate as efficiently as pp65-negative PMN as indicated by the comparable relative proportion of positive cells before and after migration. Infectious virus was recovered from PMN of six subjects (two seronegative and four seropositive) after a 24-h cocultivation with either HUVEC or HELF-infected cells. HCMV was isolated both on HUVEC and HELF monolayers in comparable amounts. Mean number of p72-positive cells detected by the shell vial assay was $49.4$ (range 2–300)/10^3 PMN inoculated. On the other hand, supernatants from the last washing, which were inoculated in parallel with migrated PMN, were negative for virus isolation.

Viral DNA was detected in each aliquot of migrated PMN with a mean number of genome equivalents of $3 \times 10^6$ (range $10^5–10^7$/10^6 PMN. The amount of viral DNA did not significantly change after deoxyribonuclease treatment before extraction (data not shown).

In some experiments, migrated PMN were further purified by FACS® and sorted population examined for presence of pp65, infectious virus, and DNA. The same parameters were determined before FACS® purification. Results of a representative experiment are reported in Table II, which shows that grossly comparable levels of antigenemia and leukoDNAemia were detected in migrated PMN before and after FACS® purification, whereas, for viremia, levels of infectious virus were slightly decreasing.

Contact-dependent uptake of pp65 by PMN cocultured with HCMV-infected HUVEC. To assess whether intercellular contact was required for pp65 uptake by PMN from HCMV-infected EC monolayers, we used three independent approaches. First, no pp65-positive PMN was detected after a 24-h coculture, when contact with HCMV-infected HUVEC or HELF monolayers (96 h p.i.) was prevented by trans-well inserts. Second, anti-LFA-1 (CD18) antibody decreased the number of pp65-positive PMN from 1486 to 216 (85.46% reduction). In addition, the mAb anti–ICAM-1 reduced the number of pp65-positive PMN from 1607 to 365$\pm$256.7 (77.28% reduction). However, whereas the level of the blocking effect observed with CD18 mAb was more consistent (66.6–93% reduction), that of anti–ICAM-1 was more variable (25–92.5% reduction). Finally, after a 1-h coculture with infected EC, nonadherent PMN were carefully and extensively removed by repeated washings of the coculture, followed by coculturing of the adherent fraction of PMN for an additional 24 h. To identify PMN, we used a flow cytometry approach with a predetermined setting for the separation of PMN from EC based on physical parameters (Fig. 3). The results of these experiments are reported in Table III, which shows that: (a) the percentage of pp65-positive cells dramatically increased with time in the adhering fraction of PMN, and (b) pp65-positive PMN recovered in suspension after a 24-h coculture represent PMN initially adhering to and subse-

Table II. Results of Viral Parameters Determined on Migrated PMN Fractions before and after FACS® Purification

<table>
<thead>
<tr>
<th>PMN fraction following</th>
<th>pp65+</th>
<th>Infectious virus/DNA</th>
<th>DNA</th>
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<tbody>
<tr>
<td>Migration to FMLP</td>
<td>$2 \times 10^5$</td>
<td>49</td>
<td>$5 \times 10^5$</td>
</tr>
<tr>
<td>Migration to FMLP and FACS® purification</td>
<td>$2 \times 10^5$</td>
<td>7</td>
<td>$\geq 10^6$</td>
</tr>
</tbody>
</table>

*No. of HCMV pp65-positive PMN/2×10^6 examined (pp65 antigenemia). 1 No. of HCMV p72-positive HELF nuclei/2×10^6 PMN inoculated (viremia). 2 No. of HCMV genome equivalents/10^6 PMN examined (leukoDNAemia).
quently detaching from HCMV-infected EC. Thus, transient adhesion of PMN to HCMV-infected EC does occur and appears essential for pp65 uptake.

EM. Complete (enveloped) virus particles were detected by EM in the cytoplasm of some PMN after a 24-h coculture (Fig. 4). However, more often, naked particles were observed either within vacuoles or not (not shown). In addition, phagocytosis of dense bodies as well as presence of dense bodies inside cytoplasmic vacuoles of PMN was often observed (Fig. 5).

### Discussion

The most important findings reported in the present study are the following: (a) pp65 is detected in PMN cocultured in vitro with EC infected with a HCMV clinical strain recovered from blood and propagated in EC; (b) pp65 uptake does also occur when PMN are cocultured with HELF cells infected with low-passage HCMV isolates recovered from different body sites; (c) the phenomenon was never observed with PMN cocultured with HELF infected with laboratory-adapted AD169, Davis, and Towne HCMV strains or with PMN incubated with cell-free suspension of either AD169 or low-passage clinical strains; (d) PMN cocultured with HCMV-infected HUVEC or HELF cells are capable of transmitting infectious virus when cocultured with fresh HUVEC or HELF cells; (e) cocultured PMN carry high levels of viral DNA. Optimal time for PMN

<table>
<thead>
<tr>
<th>PMN fraction</th>
<th>% pp65-positive PMN after time of coculture</th>
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<tbody>
<tr>
<td></td>
<td>1 h</td>
</tr>
<tr>
<td>Adhering</td>
<td>5.70</td>
</tr>
<tr>
<td>Nonadhering</td>
<td>1.25</td>
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</table>

*Reported data are representative of three different experiments.

![Figure 4. Ultrastructure of a PMN (right) and a portion of an HELF cell (left) after cocultivation. The insert shows enveloped virus particles (arrows) in both cells. Bar, 0.8 μm.](image1)

![Figure 5. Multiple dense bodies are either (A) in the process of being taken up or (B) are present in the cytoplasm of a PMN (white arrows). Bar, 1.1 μm.](image2)
cocultivation is 24 h because after a 48–96-h cocultivation the number of viable and migrating PMN progressively decreases.

Our data provide evidence that in vivo and in vitro PMN–EC interactions may be similar as suggested by the following events: (a) upon adhesion to HCMV-infected EC, a low percentage of PMN (0.2–5%, a frequency comparable to that observed in vivo in patients with the highest antigenemia levels) acquire pp65 that localizes in the nucleus, as observed in ex vivo cytoospin preparations; (b) pp65-positive PMN detach from infected EC and can be recovered in coculture medium (corresponding to the in vivo pp65 antigenemia detected in circulating PMN); (c) PMN can take up and transmit infectious virus from infected to uninfected EC or HELF (corresponding to the in vivo viremia); (d) cocultured PMN carry viral DNA (corresponding to the in vivo leukoDNAemia). In addition, during cocultivation, infected HUVEC may detach from the cell monolayer and be released together with cocultured PMN in the medium. This event perfectly mimics what has been observed in vivo in patients with disseminated HCMV infection and organ involvement, in whom coculturing HCMV-infected EC become detectable inuffy coat samples examined for pp65 antigenemia (6, 7).

It has been reported that PMN adherence to HCMV-infected EC significantly increases by 24 h p.i. and is not induced by soluble components released in the medium by virus-infected cells (14, 15). More recently, Sedmak et al. showed that HCMV infection substantially upregulates the expression of the adhesion molecule ICAM-1 on infected EC (16), and the same appears to occur in HCMV-infected HELF (17). Thus, major interactions between HCMV-infected EC (or HELF) and PMN may involve this molecule and its known ligands, LFA-1 and MAC-1, which belong to the CD11/18 integrin subfamily and are predominantly found on the surface of PMN (12). In agreement with these data, our results indicate that adherence of PMN to infected monolayers does occur and is required for uptake of pp65. In fact, when cell to cell contact was physically (membrane insert) or immunologically (anti-CD18, anti–ICAM-1) prevented, pp65-positive PMN were absent or greatly reduced in number, respectively. Moreover, we showed that uptake of pp65 increases dramatically in PMN initially adhering to and subsequently detaching from HCMV-infected EC. Contact or adherence to HCMV-infected EC has also been shown to be necessary for monocyte infection (18).

The most intriguing aspect of our study is the dramatic difference observed between HCMV laboratory–adapted strains and low-passage clinical strains with respect to pp65 and infectious virus uptake by PMN. AD169 and Towne were developed as vaccine candidates and were serially passaged in HELF with the intent of attenuating their virulence before being used in clinical trials (19, 20). The ability of highly passaged laboratory strains to infect and replicate in EC has been reported to be markedly impaired for AD169 and Towne (21, 22), and absent for Davis (23). Moreover, 20 passages of a clinical HCMV strain on HELF were sufficient to cause definite alterations in its cytopathogenicity and replicative capacity in EC (21). Our own experience supports the above reported findings, that in all laboratory strains examined in the present study grew very poorly or not at all in EC.

Moreover, dramatic interstrain differences have been reported between attenuated (Towne and AD169) and a low-passage (Toledo) strain of HCMV, regarding the ability to rep-


