In Vitro Model for the Study of the Dissociation of Increasing Antigenemia and Decreasing DNAemia and Viremia during Treatment of Human Cytomegalovirus Infection with Ganciclovir in Transplant Recipients

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The paradox phenomenon (i.e., the dissociation of increasing antigenemia and decreasing DNAemia and viremia) that occurs during treatment of human cytomegalovirus (HCMV) infections with ganciclovir (Gcv), in transplant recipients, was investigated by use of an in vitro model for the study of interactions between polymorphonuclear leukocytes and endothelial cells. The paradox phenomenon was reproduced in vitro in the presence of Gcv and, to a much lesser extent, in the presence of cidofovir, but not in the presence of foscarnet. The pathogenetic basis for such a paradox response was found, by use of drug concentrations in the range of 90%-99% of the inhibitory dose, to rely on the partial synthesis of HCMV phosphoprotein 65. The opposite situation (i.e., the simultaneous increase of antigenemia, viremia, and DNAemia), which is observed in clinical conditions associated with inefficacy of treatment due to drug-resistant strains, was also reproduced in vitro by use of drug-resistant HCMV strains. The conclusion for clinicians is that antiviral therapy must be changed only in the latter case.

In solid-organ transplant recipients with primary human cytomegalovirus (HCMV) infection who receive treatment with ganciclovir (Gcv), increasing levels of HCMV antigenemia have been reported repeatedly [1, 2]. In addition, similar findings have been reported for Gcv-treated hematopoietic stem-cell transplant (HSCT) recipients with HCMV infection [3, 4]. The increase in antigenemia during treatment appeared to occur more frequently when treatment with Gcv was started as soon as HCMV pp65 was detected in the blood of transplant recipients and was reported to be associated with a higher rate of HCMV infection relapses during follow-up [2]. Although foscarnet (PFA) is used to a much lesser extent, it did not seem to produce the same effects as did Gcv, when administered to similar patients. In addition, increasing or persistently high levels of antigenemia during treatment with Gcv were found to be associated with decreasing or persistently low levels of DNAemia and viremia, thus revealing an unexplained dissociation that often prompted clinicians to change treatment. However, this treatment shift was mostly unjustified, particularly in cases when viremia (i.e., detection of infectious virus in blood) was negative, thus documenting the efficacy of treatment with Gcv in blocking virus replication. Therefore, the anomalous diagnostic finding reported above was often misleading to clinicians in transplantation centers where antigenemia-guided preemptive treatment was used.

Except for diagnostic assays that measure plasma levels of viral DNA released by infected endothelial cells

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or leukocytes undergoing lysis, all the other HCMV diagnostic assays are based on the presence of virus and viral products in circulating leukocytes, particularly polymorphonuclear leukocytes (PMNLs) and monocytes. Leukocytes are thought to uptake virus and viral products from infected endothelial cells by means of transitory microfusion events that require previous attraction and adhesion, as reported elsewhere [5]. Recently, an in vitro model for the study of interactions between HCMV-infected endothelial cells and peripheral blood leukocytes (PBLs; i.e., PMNLs and monocytes) was developed [6]. This model allows in vitro generation of antigenemia, viremia, DNAemia, and RNAemia starting from peripheral blood leukocytes from healthy blood donors. Using this model, we investigated the reported dissociation between levels of antigenemia and those of DNAemia and viremia, by coculturing PMNLs and endothelial cells infected with clinical HCMV isolates in the presence or absence of Gcv and other antiviral drugs. When used at a dose comparable to plasma concentrations reached during therapy, Gcv reproduced in vitro the increasing antigenemia levels observed in vivo, concomitantly with stable or decreasing levels of DNAemia and viremia [7, 8]. The in vitro study of the kinetics of pp65 synthesis in infected endothelial cells, untreated or treated with antiviral drugs, significantly contributed to clarifying the mechanism of the previously unexplained dissociation. However, in the clinical setting, increasing antigenemia during treatment with Gcv may or may not be associated with increasing DNAemia and viremia. The therapeutic approach in the 2 different clinical situations is completely different, because antiviral therapy must not be changed in case of dissociation.

MATERIALS AND METHODS

**Virus strains.** Two drug-sensitive HCMV clinical isolates recently recovered from patients who were naive for HCMV-specific antiviral drugs were used to infect human umbilical vein endothelial cells (HUVECs). One of these strains (VR6110) was recovered from PBLs of a patient with AIDS [6], whereas the other strain (VR1814) was recovered from cervical secretions of an immunocompromised pregnant woman [9]. Both strains, which had mean Gcv ID₅₀ values of ≤3.0 μmol/L in human embryonic lung fibroblasts (HELFS), were adapted to growth in HUVECs, as reported elsewhere [9]. In addition, 2 drug-resistant HCMV clinical isolates were assayed on HUVECs. The first strain (VR5747), which was recovered from PBLs of a heart-transplant recipient treated with 3 induction courses of intravenous Gcv followed by a 2-month maintenance treatment with oral Gcv, had a Gcv ID₅₀ value of 25 μmol/L in HELFS and a mutation in UL97 (C607Y) that is responsible for resistance to Gcv [10]. The second strain (VR4760), which was recovered from PBLs of a patient with AIDS who was treated with multiple induction courses of both Gcv and PFA followed by combined therapy with both drugs at full dosage, had a Gcv ID₅₀ value of 40 μmol/L and a PFA ID₅₀ value of 514 μmol/L in HELFS; the strain had a UL97 mutation (M460I) that was responsible for resistance to Gcv and a UL54 mutation (V715M) that was responsible for resistance to PFA [11, 12].

**HUVEC cultures.** HUVECs were obtained by treating veins from umbilical cords, obtained from infants delivered by caesarean section, with trypsin. Medium for HUVEC culture was endothelial cell growth medium–2 (Clonetics Bio-Whittaker) supplemented with 2% fetal calf serum. Cells were used at passages 2–5 and were derived from multiple batches obtained from different umbilical cords. The lack of HCMV infection in HUVECs as a potential result of unrecognized congenital HCMV infection was routinely verified by nested polymerase chain reaction (PCR) [6].

**Adaptation of HCMV isolates to growth in HUVECs.** The process of adaptation of VR6110 and VR1814 to growth in HUVECs has been reported elsewhere [9]. The HUVEC adaptation of the 2 resistant HCMV strains followed the same protocol used for susceptible strains. In brief, HELFS were first infected with each strain at an MOI of 1–5. When showing 100% cytopathic effect, infected HELFS were trypsinized and inoculated 1:3 onto confluent monolayers of uninfected HUVECs. After 7 days, infected HUVECs were trypsinized and mixed 1:2 with uninfected HUVECs. This procedure was repeated until passage 6, when HUVECs were sonicated and cell-free virus was propagated. Virus growth in HUVECs was checked at each passage by immunofluorescence with monoclonal antibodies (MAbs) to the major immediate-early (IE) proteins p72 or gB. MAbs to gB were provided by Dr. Lenore Pereira (University of California, San Francisco). Using these HUVEC-adapted drug-sensitive and –resistant HCMV strains, we could study their interaction with PMNLs in the presence or absence of different drugs.

**PMNL coculture and purification.** To reproduce in vitro a condition as close as possible to that in vivo, interactions between HUVECs and PMNLs were studied. PMNL preparations from healthy blood donors were obtained by dextran concentration followed by ficoll-hypaque separation [6] and were cocultured overnight with infected HUVEC monolayers, 24–168 h after infection, in either the presence or absence of antiviral drugs (see below). Cocultured PMNL suspensions containing HUVECs detached from the growth surface were then placed for 3 h at 37°C in the upper compartment of a cell-culture device separated by a Transwell filter (5-μm pore size; Costar) from the lower compartment, which contained 10⁻⁴ mol/L N-formyl-Met-Leu-Phe-Ala (Sigma), according to a procedure reported elsewhere [6]. This procedure causes PMNLs to migrate to the lower compartment, where they are
separated from contaminating HUVECs, reaching a level of purity (99%) similar to that achieved by fluorescence-activated cell sorting [6].

**Antiviral drug susceptibility testing on HUVECs and determination of the in vitro working drug concentration.** The following antiviral drugs currently used in clinical practice were tested: Gcv (Roche Products), PFA (Astra Zeneca), and cidofovir (Cdv; Gilead Sciences). In addition, a new compound, 2-bromo-5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (BDCRB; provided by Dr. John Drach, University of Michigan, Ann Arbor), which inhibits HCMV DNA maturation and packaging, was studied for comparison [13, 14]. ID$_{50}$ values of Gcv, PFA, Cdv, and BDCRB, previously determined on HCMV strains growing in HELFs, were recalculated for the same 4 strains growing in HUVECs. Thus, the actual drug concentrations used in all in vitro experiments (Gcv, 20 μmol/L; PFA, 400 μmol/L; Cdv, 5 μmol/L; and BDCRB, 30 μmol/L) were in the range of ID$_{50}$ to ID$_{90}$ as determined on HUVECs. Working drug doses used in vitro were higher than the mean and close to the peak concentrations reached in vivo in plasma during therapy [15–17]. Susceptibility of HCMV to drugs was determined by use of an IE plaque reduction assay, as described elsewhere [18].

**Kinetics of HCMV infection in HUVEC cultures and PMNL suspensions cocultured in the presence or absence of Gcv or other antiviral drugs.** After infection with each of the 4 HCMV strains at an MOI of 0.01–0.1, HUVEC cultures were stained daily, 24–168 h after infection, for the IE antigen p72, the early-late protein pp65, and the late glycoprotein gB, by use of immunofluorescence and the relevant MAbs. In parallel, infected cell culture medium was serially collected for quantification of cell-free virus released from infected cells. In addition, after coculture, PMNL suspensions were collected daily, purified as described elsewhere [6], and aliquoted for quantification of pp65-positive PMNLs, PMNLs carrying infectious virus, and viral DNA transported by PMNLs, thus mimicking antigenemia, viremia, and DNAemia, respectively.

**Virus assays on PMNL suspensions.** The number of pp65-positive PMNLs was determined on PMNL cytospin preparations (1 x 10$^5$ cells) that were fixed and stained according to a procedure described elsewhere [19, 20]. The number of PMNLs carrying infectious virus was quantified by inoculating 1 x 10$^5$ PMNLs onto HELF monolayers grown in shell vials and counting the number of p72-positive HELF nuclei, after staining with an anti-p72 MAB 16–24 h after infection [21]. HCMV DNA was quantified in PMNL samples (1 x 10$^5$ cells) by quantitative PCR, as reported elsewhere [22, 23].

**RESULTS**

*In vitro experiments to study the interaction between infected HUVECs and PMNLs in the presence or absence of Gcv or other antiviral drugs.* To approximate the interactions that occur in vivo between HCMV-infected endothelial cells and PMNLs, we used the in vitro model described above, which has been shown to enable transfer of virus and viral products to PMNLs via transitory microfusion events [5]. However, to investigate the pathogenetic mechanism of increasing antigenemia during treatment with Gcv in vivo, infected HUVECs were treated, after infection and before coculture with PMNLs, with Gcv (the antiviral drug currently reported to give increasing antigenemia in vivo) and with PFA (which has a mechanism of action different from that of Gcv, since it competes with pyrophosphate instead of nucleoside) and Cdv (which has a mechanism of action similar to, but distinct from, that of Gcv, since Cdv is already phosphorylated). In addition, the new, experimental drug BDCRB was tested. The in vitro model allowed an approximate reproduction in vitro of the onset of primary infection in vivo, where antiviral treatment acts in the absence of a preexisting immune response. In addition, infection of HUVECs with Gcv-resistant and Gcv-and-PFA–resistant strains permitted investigation of the kinetics of increasing levels of the different diagnostic parameters during treatment.

**Kinetics of HCMV infection in HUVECs before coculture.** After inoculation of a cell-free preparation of HUVEC-adapted VR1814, the time-course of virus replication in HUVECs, in the absence and presence of Gcv or other antiviral drugs, was investigated both by titrating infectious virus released in the medium and by immunostaining cultures for p72, pp65, and gB every 24 h, from 72 to 168 h after infection. Given that infectious virus and viral products may be transferred to PMNLs only from the cytoplasm of infected cells during coculture [5], increasing levels of pp65-positive PMNLs after coculture with Gcv-treated HUVECs could only be caused by the presence of pp65 in the cytoplasm of infected HUVECs. In control HUVEC cultures infected at an MOI of ∼0.1, p72 was detected in nuclei of infected cells starting 24 h after infection, whereas pp65 (nuclear and cytoplasmic) and gB (cytoplasmic and paranuclear) started appearing 72 h after infection. As shown in figure 1A, 1D, 1G, and 1J, at 96 h after infection, infected cell foci consisted of single infected cells, if stained for gB, or plaques, if stained for either the IE antigen p72 or the matrix protein pp65. In more detail, gB-stained cells were markedly fluorescent over the entire cytoplasm, including the thin cytoplasmic bridges, with the area of most intense staining restricted to the Golgi zone. Plaques of pp65-stained cells were subconfluent and consisted of a central parental infected cell, mostly fluorescent over the entire cytoplasm and surrounded by numerous cells with pp65-positive nuclei slightly declining in intensity of fluorescence towards the periphery (figure 2A). In contrast, plaques of p72-stained cells were much smaller and well separated from one another, consisting of markedly stained...
Figure 1. Human umbilical vein endothelial cell (HUVEC) cultures infected with a ganciclovir (Gcv)-sensitive human cytomegalovirus (HCMV) strain (VR1814) 96 h after infection in the absence of antiviral drugs (A, D, G, and J) and in the presence of Gcv (B, E, H, and K) or foscarnet (PFA) (C, F, I, and L). Cultures were fixed and stained with monoclonal antibodies to immediate-early (IE) protein p72 (A–C), both p72 and gB (D–F), gB alone (G–I), and pp65 (J–L). With respect to control cultures, Gcv showed a few smaller IE plaques (B), each generated by a gB-stained parental infected cell (E); cells less markedly stained for gB (H); and much smaller pp65 plaques surrounding the parental infected cell (K). PFA showed the same trend as did Gcv, with more marked inhibition of virus replication (C, F, I, and L).

p72 nuclei surrounding parental infected cells displaying nuclei larger in size (figure 2D).

When infected HUVEC cultures were treated with Gcv, the number of infected foci decreased drastically, to 1%–5%, but did not disappear at 96 h after infection, whereas the expression of viral proteins within each infected focus was markedly reduced (figure 1B, 1E, 1H, and 1K). In fact, gB-stained cells showed staining restricted to the Golgi zone, whereas plaques of pp65 and IE antigens were markedly smaller (figure 2B and 2E). However, despite treatment with Gcv, in pp65 plaques, parental infected cells showed abundant presence of pp65 over the entire cytoplasm, thus being suitable for transfer to PMNLs during coculture. When PFA was used to block virus replication in HUVEC cultures, the number of infected foci was further reduced, to 0.1%–0.5% (figure 1C, 1F, 1I, and 1L), and the number of cells showing the presence of pp65 (susceptible to be transferred to PMNLs during coculture) in the cytoplasm (figure 2C) and p72 in the nucleus (figure 2F) was negligible. The effect of Cdv was intermediate between those of Gcv and PFA; however, HUVEC cultures treated with BDCRB were comparable to untreated cultures, although expression of viral proteins appeared to be slightly reduced (data not shown).

Infection of HUVECs with either Gcv-resistant or Gcv-and-PFA–resistant strains showed a kinetics of HCMV infection that was similar to that of untreated control cultures but that was only slightly delayed in the presence of drug-inducing resistance. In particular, although the amount of infectious virus released by infected cells, in the presence of antiviral drugs, was negligible when testing HCMV drug–susceptible strains, the virus yield of HUVEC cultures infected with resistant strains, in the presence of the relevant drug-inducing resistance, was close to that of control cultures. This phenomenon explains why, in the case of drug-susceptible strains, only pp65 was transferred to PMNLs during coculture, whereas, in the case of drug-resistant strains, infectious virus and viral DNA, in addition to pp65, were transferred to PMNLs (see below).

The kinetics of virus yield released in the medium of treated and untreated HUVEC cultures is reported in figure 3. Although, at 96 h after infection, cell-free virus reached a peak of >10^6 focus-forming units (ffu)/mL in control cultures and only 10^5 ffu/mL in Gcv-treated cultures, the amount of virus detectable in PFA-treated cultures was negligible. The same trend was found to persist until 144 h after infection, when virus titer reached 3.4 x 10^6 ffu/mL in control cultures, 6 x 10^5 ffu/mL in Gcv-treated cultures, and 6.5 x 10^5 ffu/mL in PFA-treated cultures. Results comparable to those obtained with PFA were observed in Cdv- and BDCRB-treated cultures.

Kinetics of virus and viral products in PMNLs cocultured with HUVECs infected with drug-susceptible or drug-resistant HCMV strains. Overnight coculture of PMNLs and HUVECs infected with a drug-susceptible HCMV strain (VR1814) yielded a progressively increasing number of pp65–positive PMNLs, starting 48–72 h after infection and reaching the peak 120 h after infection. Subsequently, the number of pp65–positive PMNLs progressively decreased until 168 h after infection. In parallel, after coculture with HUVECs infected with the same strain and
treated with Gcv, the number of pp65-positive PMNLs showed a similar trend, yet delayed by 24 h (i.e., starting at 96 h and reaching the peak 120 h after infection) then declining. This appeared to be the in vitro basis for the reported increasing levels of antigenemia in Gcv-treated primary HCMV infections of transplant recipients. Cdv showed a trend similar to that of Gcv, although to a much lesser extent. On the contrary, coculture of PMNLs with infected HUVECs treated with PFA yielded a negligible number of pp65-positive PMNLs at 120 h after infection. Finally, BDCRB showed a kinetics of pp65-positive PMNLs comparable to that of control cultures (figure 4A).

Control HUVEC cultures showed peaks of cocultured PMNLs carrying either infectious virus (figure 4D) or viral DNA at 120 h after infection (figure 4G). After coculture, no PMNLs carrying infectious virus were found with any treatment (correlate of in vivo viremia) (figure 4D). In addition, cocultured PMNLs only evidenced background levels of viral DNA with each drug tested (correlate of in vivo DNAemia) (figure 4G).

Entirely overlapping results were observed with the other drug-susceptible HCMV strain (VR6110) tested, with regard to the number of pp65-positive PMNLs, as well as levels of infectious virus and viral DNA carried by PMNLs (data not shown). When HUVEC cultures were infected with a Gcv-resistant HCMV strain (VR5747), coculture gave curves of
pp65-positive PMNLs, as well as infectious virus and viral DNA, comparable to those of control HUVEC cultures and cultures treated with Gcv, whereas inhibitory curves similar to those obtained when we used Gcv-susceptible strains were observed in the presence of Cdv, PFA, and BDCRB (figure 4). Finally, when HUVEC cultures were infected with a Gcv- and PFA-resistant strain (VR4760), coculture showed levels of pp65-positive PMNLs, as well as infectious virus and viral DNA in PMNLs, that were comparable to control cultures and cultures treated with Gcv or PFA, whereas cultures treated with Cdv and BDCRB showed inhibitory effects similar to those observed when we used drug-susceptible HCMV strains (figure 4G, 4F, and 4I).

**DISCUSSION**

The study of the mechanism underlying the repeatedly reported paradox phenomenon of increasing levels of HCMV pp65 antigenemia dissociated from a similar trend of viremia and DNAemia in transplant recipients with Gcv-treated primary HCMV infection was the primary objective of the present study. This phenomenon occurs most often during primary HCMV infections in solid-organ transplant recipients or during HCMV infections in HSCT recipients. The major reason for this atypical response is likely to be the lack of preexisting HCMV-specific immune response in seronegative or myeloablated transplant recipients in whom the cell-mediated immune response (particularly to HCMV pp65 expressing cells) is further impaired and delayed by the immunosuppressive regimen.

We decided to investigate the paradox phenomenon in vitro, at least in the initial phase, when the in vivo conditions may be better simulated. We decided to study, in addition to Gcv, a drug with a similar structure and mechanism of inhibition of viral DNA synthesis (Cdv) and a drug with a different structure and that targets a different step in viral DNA synthesis (PFA). Both drugs are widely used in clinical practice, although they are not yet reported to be associated with a paradox response, as is Gcv. Finally, a new drug, BDCRB, which is not yet licensed for clinical use and has a different mechanism of action, was tested for comparison [13, 14]. Results of our in vitro study allow clarification of the mechanism of the paradox phenomenon, showing that Gcv and, to a lesser extent, Cdv reproduce increasing levels of antigenemia in vitro similar to those observed in vivo with Gcv [7, 8], whereas PFA substantially does not.

Because virus and viral products detected in PMNLs during performance of different diagnostic assays (antigenemia, viremia, and DNAemia) are transferred from endothelial cells to leukocytes via multiple transitory microfusion events [5], it was first necessary to carefully study the kinetics of HCMV infection in HUVECs in the presence or absence of antiviral drugs. Coculture of HUVECs and PMNLs was aimed at simulating interactions occurring between PMNLs and endothelial cells of vessel walls. Results show that increasing numbers of pp65-positive PMNLs are observed in the presence of Gcv, Cdv, and BDCRB but not in the presence of PFA. This trend was not observed for the presence of infectious virus and viral DNA in PMNLs (figure 4A, 4D, and 4G). This could be explained by the finding that, at 96 h after infection, ~1%–5% of infected cells in Gcv-treated cultures escaped blocking of viral DNA replication (figure 2B). Thus, in this aliquot of cells, assembly and maturation of dense bodies and virus particles, migration through cytoplasm, and final egress from the cell could occur [24]. At that time, the cells were rich in pp65 (dense bodies) in the cytoplasm and were responsible for the transfer of pp65 to PMNLs in Gcv-treated cultures, albeit to a lesser degree than in control cultures.

However, an important finding was that, at 96 h after infection, in control cultures, the amount of pp65 (dense bodies) released from infected cells was markedly greater than the number of infectious virus particles (figure 2). In fact, although discrete IE protein plaques were formed by each parental infected cell, leaving ~90% of the cell monolayer uninfected (figure 1A and figure 2D), almost all the cells showed the presence of pp65 inside the nucleus (figure 1F and figure 2A). It has been documented that dense bodies consist mostly of pp65 [25–27] and enter cells only when enveloped, by the same mechanism as virions [28]. Thus, the presence of pp65 inside the nucleus of all cells documents unequivocally that, at 96 h after infection, many more enveloped dense bodies than virions had already been released. This conclusion is confirmed by data obtained in Gcv-treated cultures, where, although the number
of parental infected cells is reduced to 1%–5% of controls and IE plaques involve a minor part of the cell monolayer, a much larger number of cells shows nuclear pp65. Thus, even in this condition, where viral replication is inhibited by a Gcv concentration greater than that used in vivo, the amount of enveloped dense bodies released and entering uninfected cells is sufficient to involve most of the cell monolayer. The inhibitory effect of PFA was much greater, although used at a concentration comparable with that of Gcv, as shown by the very low number of parental infected cells releasing very low amounts of infectious virus (IE plaques of 2–3 cells) in association with large but well-delimited plaques of nuclear pp65. As a consequence, the number of infected HUVECs releasing and accumulating dense bodies in the cytoplasm was too small (<0.1%) to generate a quantifiable number of pp65-positive PMNLs during coculture [6].

In parallel, the amount of infectious virus released in the medium by treated, infected HUVECs was ∼2 log₁₀ lower than that released by controls treated with Gcv and was negligible in HUVECs treated with PFA. It has recently been shown that murine cytomegalovirus, by means of a viral gene product (M50/p35) inserted into the inner nuclear membrane and interacting with a second viral protein (M53/p38) localized in the nucleus, recruits host-cell protein kinase C to the nuclear membrane. Here, the kinase increases phosphorylation of the nuclear lamins. This causes partial disruption of the lamin network, thereby facilitating capsid envelopment at the inner nuclear membrane [24], as has already been reported for herpes simplex virus [29, 30]. In this respect, it can be argued that, only when viral products dissolving the nuclear lamina are present, nucleocapsids and dense bodies may start their egress from nucleus through the nuclear lamina and the inner nuclear membrane [24, 31].

UL83, the HCMV open-reading frame coding for pp65, is known to be an early-late or γ₁ gene capable of transcribing and translating pp65, even in the presence of a viral DNA inhibitor [32]. Because of its 2 nuclear localization signals [33, 34], newly synthesized pp65 is immediately transferred to the nucleus of infected cells, where it accumulates. On the basis of the results of the present study, it appears that dense bodies
were released from the nucleus and entered uninfected cells to a much greater extent than did virions, involving both contiguous and distant cells. The same trend, to a lesser extent, was observed in Gcv-treated cultures and, to an even lesser extent, in PFA-treated cultures.

However, only parental infected cells with cytoplasmic pp65 were able to generate pp65-positive PMNLs. Thus, increasing levels of pp65 antigenemia in Gcv-treated cultures were due to the interaction of PMNLs with the fair number of infected HUVECs, in which virus replication was not inhibited, and with accumulation of pp65 in the cytoplasm. This event did not occur in PFA-treated cultures, in which the number of cells undergoing virus replication was minimal. Viral DNA did not overcome background levels in cocultured PMNLs at any time with any drug. Since transfer of viral DNA to PMNLs is essentially due to transfer of virus particles [5], the amount of infectious virus and, thus, of viral DNA transferred to PMNLs was too little to be detected. In fact, survival of infectious virus in PMNLs is short lived [5].

In conclusion, we have elucidated the mechanism underlying increasing antigenemia during Gcv-treated HCMV infections of transplant recipients by use of an in vitro model of coculture of infected HUVECs and PMNLs developed for the generation of pp65-positive PMNLs. The lack of concomitant infectious virus and increasing levels of DNA in PMNLs were found in PFA-treated cultures. Viral DNA did not occur in PFA-treated cultures, in which the number of cells undergoing virus replication was minimal. Viral DNA did not overcome background levels in cocultured PMNLs at any time with any drug. Since transfer of viral DNA to PMNLs is essentially due to transfer of virus particles [5], the amount of infectious virus and, thus, of viral DNA transferred to PMNLs was too little to be detected. In fact, survival of infectious virus in PMNLs is short lived [5].

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