Cathepsins are involved in virus-induced cell death in ICP4 and Us3 deletion mutant herpes simplex virus type 1-infected monocytic cells

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We have studied cell death and its mechanisms in herpes simplex virus type 1 (HSV-1)-infected monocytic cells. The HSV-1 ICP4 and Us3 deletion mutant, d120 caused both apoptosis and necroptosis in d120-infected monocytic cells. At a late time point of infection the number of apoptotic cells was increased significantly in d120-infected cells when compared with uninfected or parental HSV-1 (KOS)-infected cells. Necroptosis inhibitor treatment increased the number of viable cells among the d120-infected cells, indicating that cell death in d120-infected cells was, in part, because of necroptosis. Moreover, lysosomal membrane permeabilization and cathepsin B and H activities were increased significantly in d120-infected cells. Inhibition of cathepsin B and S activities with specific cathepsin inhibitors led to increased cell viability, and inhibition of cathepsin L activity resulted in a decreased number of apoptotic cells. This indicates that cathepsins B, L and S may act as cell-death mediators in d120-infected monocytic cells. In addition, caspase 3 activity was increased significantly in d120-infected cells. However, the caspase 3 inhibitor treatment did not decrease the number of apoptotic cells. In contrast, inhibition of cathepsin L activity by cathepsin L-specific inhibitor clearly decreased caspase 3 activity and the number of apoptotic cells in d120-infected cells. This might suggest that, in d120-infected monocytic cells, cathepsin L activates caspase 3 and thus mediates d120-induced apoptosis. Taken together, these findings suggest that d120-induced cell death is both apoptotic and necroptotic.

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) is a common pathogen that replicates in a variety of cell types during acute infection (Roizman et al., 2007). After lytic infection HSV-1 remains latent in the neurons of its host for life and can reactivate to cause lesions at or near the initial site of infection. Like other herpesviruses, HSV-1 expresses a large number of enzymes involved in nucleic acid metabolism, DNA synthesis and the processing of proteins. Productive viral infection is accompanied by inevitable cell destruction. HSV-1 has several strategies to combat the responses of the infected host, among them the prevention of the blocking protein kinase R-mediated shut-off of host protein synthesis (He et al., 1997), having a latent form of infection with no protein expression (Roizman et al., 2007), blocking presentation of antigenic peptides on the cell surface (Fruh et al., 1995; Hill et al., 1995) and blocking apoptosis (Galvan & Roizman, 1998, 2001). Other proteins reported to participate in blocking apoptosis are, for example, the immediate–early protein ICP4 (Aubert & Blaho, 1999; Leopardi & Roizman, 1996), the essential viral glycoprotein D (Zhou et al., 2000) and the late glycoprotein Us5 (Jerome et al., 1999). In addition, the HSV-1 latency-associated transcript has a pro-survival function as this RNA transcript can inhibit apoptosis (Ghiasi et al., 2002; Li et al., 2010; Perng et al., 2000; Thompson & Sawtell, 2001).

The extent of apoptosis observed following HSV-1 infection is cell-type-dependent, indicating that HSV-1-induced apoptosis is regulated by different cellular factors such as caspases, Bcl-2 family members and nuclear factor κB (Ahmed et al., 2002; Asano et al., 2000; Aubert & Blaho, 2003; Branco & Fraser, 2005; Galvan & Roizman, 1998; Adachi, 1997). However, HSV-1 is able to block apoptosis at multiple stages of infection to prevent the host cell from dying prematurely (Aubert & Blaho, 1999; Galvan & Roizman, 1998; Koyama & Miwa, 1997). For example, the late protein kinase Us3 contributes to blocking HSV-1-induced apoptosis (Leopardi et al., 1997; Munger & Roizman, 2001). Other proteins reported to participate in blocking apoptosis are, for example, the immediate–early protein ICP4 (Aubert & Blaho, 1999; Leopardi & Roizman, 1996), the essential viral glycoprotein D (Zhou et al., 2000) and the late glycoprotein Us5 (Jerome et al., 1999). In addition, the HSV-1 latency-associated transcript has a pro-survival function as this RNA transcript can inhibit apoptosis (Ghiasi et al., 2002; Li et al., 2010; Perng et al., 2000; Thompson & Sawtell, 2001).

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Nguyen et al., 2007). Caspases 3 and 9 have been reported to mediate HSV-1-induced apoptosis in human epithelial HEp-2 cells (Aubert et al., 2007; Kraft et al., 2006) whereas caspase 8 has been shown not to mediate HSV-1-induced apoptosis (Aubert et al., 2007). Thus HSV-1 induces apoptosis through the intrinsic pathway. The late protein kinase Us3 has been shown to block caspase 3 activation (Benetti & Roizman, 2007; Hagglund et al., 2002) and the caspase-dependent double cleavage and activation of the pro-apoptotic Bcl-xl/Bcl-2 associated death promoter (Benetti et al., 2003).

Apoptosis is a caspase-dependent type of programmed cell death, in which the activated caspases cleave their substrates leading inevitably to cell death. However, there is also a caspase-independent type of programmed cell death called necroptosis. Necroptosis, or programmed necrosis, has been described recently and shares some regulatory mechanisms with apoptosis (Declercq et al., 2009; Degterev et al., 2005; Tait & Green, 2008), but the proteins involved in necroptosis are not fully known. However, it has been shown that the receptor-binding proteins RIP1 and RIP3 kinase regulate the decision between necroptosis and survival together (Declercq et al., 2009). The murine cytomegalovirus inhibitor of RIP activation (vIRA) targets RIP3, thus vIRA interferes with RIP1–RIP3 interactions characteristic of necroptosis (Upton et al., 2010).

Lysoosomal cysteine proteases, cathepsins, are papain-like enzymes that degrade proteins in lysosomes at lower pH. Cathepsins can also act at neutral pH outside lysosomes. Their survival times at neutral pH range from several minutes (cathepsin L) (Turk et al., 1993) to several hours (cathepsin S) (Kirschke et al., 1989). Cathepsins are involved, for example, in antigen processing and presentation, cytokine regulation and Toll-like receptor signalling (Colbert et al., 2009). Among these physiological functions, cathepsins mediate apoptosis induced by different stimuli (Guicciardi et al., 2004; Yamashima & Oikawa, 2009). Cathepsins are released into the cytosol due to permeabilization of the lysosomal membrane. It has been shown that there is a quantitative relationship between the amount of lysosomal rupture and the mode of cell death. Low-intensity stresses trigger a limited release of lysosomal enzymes into the cytosol followed by apoptosis, while high-intensity stresses lead to generalized lysosomal rupture and necrosis (Brunk et al., 1997; Bursch, 2001). Cathepsin B has been shown to mediate the caspase 8-dependent apoptotic signalling cascade triggered by tumour necrosis factor (TNF) in vivo (Guicciardi et al., 2000). Cathepsin B can trigger cytochrome c release from mitochondria in an in vitro system. This places cathepsin B within the TNF-triggered apoptotic cascade as an enhancer acting upstream of the mitochondria. The pro-apoptotic Bcl-2 family member Bid can be cleaved by cathepsins in a variety of cell models (Blomgran et al., 2007; Cirman et al., 2004; Stoka et al., 2001). Cathepsins B, L, S and K can cleave Bid upstream of mitochondria (Cirman et al., 2004). In addition, other pro-apoptotic targets, such as the anti-apoptotic Bcl-2 family members Bcl-2, Bcl-xl and Mcl-1, are cathepsin substrates (Droga-Mazovec et al., 2008; Turk et al., 2002). Cathepsin L is the most potent among cathepsins at degrading Bcl-2 family members. However, at neutral pH, the more stable cathepsin B plays an important role in cleaving the substrates (Droga-Mazovec et al., 2008).

In addition to apoptosis, cathepsins mediate or regulate the cellular lysosomal degradation pathway called autophagy (Uchiyama, 2001). Autophagy is a highly conserved mechanism, which plays an important role, among others, in recycling nutrients and energy, and degrading unwanted cytoplasmic constituents. In addition, autophagy is involved in innate and adaptive immunity (Levine & Deretic, 2007) and it has a critical role in defending against viral infections (Orvedahl & Levine, 2008). For example, HSV-1 infection induces autophagy but this pathway is blocked by the HSV-1 neurovirulence gene product (ICP34.5), which inactivates the essential autophagy protein Beclin-1 (Leib et al., 2009; Orvedahl et al., 2007).

We have shown previously that the ICP4 and Us3 deletion mutant of HSV-1, d120, induced cell death in monocyctic cells, mainly because of apoptosis (Peri et al., 2008). In the present study, we have characterized the d120-induced cell death in monocyctic cells further, and have studied the roles of cathepsins and caspase 3 in d120-induced cell death.

**RESULTS**

**d120, the ICP4 and Us3 deletion mutant of HSV-1, induces apoptosis and necroptosis in U937 cells**

We have shown previously that the HSV-1 ICP4 and Us3 deletion mutant d120 induces apoptosis in cultured monocyctic cells (U937) (Peri et al., 2008). In order to study whether d120 infection also induces other types of cell death, the proportions of viable, apoptotic and necroptotic cells were analysed in HSV-1-infected U937 cells.

The number of viable cells did not decrease in HSV-1-infected cells at an early time point of infection [5 h post-infection (p.i.)] when compared with uninfected cells (data not shown). However, at a late time point of infection (21 h p.i.), the number of viable cells was decreased significantly in d120-infected U937 cells compared with uninfected or parental virus [HSV-1 (KOS)]-infected cells ($P<0.008$ and $P<0.001$, respectively) (Fig. 1a). The proportion of viable cells in d120-infected cells was, approximately, only 28%. In addition, the number of viable cells was decreased notably in other Us3 deletion virus R7041-infected cells compared with uninfected, rescue virus R7306-infected or parental virus HSV-1 (F)-infected cells ($P<0.001$, $P=0.004$ and $P=0.021$, respectively) (Fig. 1a). In contrast, there was no significant
difference between the number of viable cells in HSV-1 (KOS)-, R7306- or HSV-1 (F)-infected U937 cells compared with uninfected cells (Fig. 1a).

To explore the types of HSV-1-induced cell death, HSV-1-infected U937 cells were studied for apoptosis and necroptosis. For apoptosis studies, infected U937 cells were stained with early apoptosis marker as described in Methods. At an early time point of infection, there was no difference in the number of apoptotic cells between uninfected and HSV-1-infected cells (data not shown). At a late time point of infection, the number of apoptotic cells was increased significantly in d120-infected cells compared with uninfected or parental virus HSV-1 (KOS)-infected cells (P<0.001 and P<0.001, respectively) (Fig. 1b). The proportion of apoptotic cells in d120-infected cells was approximately 46%, whereas in uninfected cells it was approximately 7% (Fig. 1b). Furthermore, the number of apoptotic cells was increased in R7041-infected cells (27%) when compared with uninfected (7%) and its rescue virus R7306-infected (10%) cells (P<0.001 and P<0.001, respectively) (Fig. 1b).

To study necroptosis, U937 cells were treated with a necroptosis inhibitor, necrostatin-1 (Nec-1), beginning 3 h before HSV-1 infection. Cell viability was studied at a late time point of infection. The number of viable cells was increased significantly in Nec-1-treated d120-infected cells when compared with untreated d120-infected cells (50 and 35%, respectively) (P<0.001) (Fig. 1c). The Nec-1 treatment did not increase cell viability in other HSV-1 infections. The Nec-1 solvent, DMSO, did not have a significant effect on cell viability (data not shown).

d120 induces lysosomal membrane permeabilization and cathepsin activation

In order to study the pathways leading to d120-induced cell death further, we measured cathepsin activities in HSV-1-infected cells. Cathepsins can trigger apoptosis by their selective translocation from lysosomes to the cytosol and nucleus. In addition, an important factor in cathepsin-triggered apoptosis is the quantitative relationship between the amount of lysosomal rupture and mode of cell death. To study lysosomal stability in d120-infected U937 cells, HSV-1-infected U937 cells were stained with acridine orange at a late time point of infection. Acridine orange accumulates in acidic cellular compartments, such as lysosomes, and fluoresces red. When released into the cytosol, acridine orange fluoresces green. To measure HSV-1-induced lysosomal membrane destabilization, acridine orange-stained U937 cells were analysed by using flow cytometry. Fig. 2 shows the percentage of permeable lysosomes (i.e. the decrease in red fluorescence) in U937 cells. The lysosomal membrane permeabilization was increased significantly in d120-infected cells when compared with HSV-1 (KOS)-infected and uninfected cells (P<0.001 and P<0.001, respectively) (Fig. 2). In addition, in R7041-infected U937 cells, the amount of lysosomal membrane permeabilization was increased significantly when compared with R7306-infected or uninfected cells (P<0.001 and P<0.001, respectively). The amount of
lysosomal membrane permeabilization was not increased in HSV-1 (KOS)-, R7306- or HSV-1 (F)-infected U937 cells when compared to uninfected cells (Fig. 2).

Since lysosomal membrane permeabilization was increased, we wanted to study whether the rupture of lysosomes resulted in increased cathepsin activities. As a result of the massive cell death in d120-infected cells, the values for cathepsin activity were proportioned to the number of viable cells. Cathepsin B activity was increased significantly in d120-infected U937 cells at a late time point of infection when compared with uninfected or parental virus HSV-1 (KOS)-infected cells (P<0.012 and P=0.019, respectively) (Fig. 3). Also, cathepsin H activity was increased significantly in d120-infected cells when compared with uninfected cells (P=0.048) (Fig. 3). There was no remarkable difference in the cathepsin B, H, L or S activities between wild-type HSV-1-infected or uninfected U937 cells at an early time point of infection (data not shown).

**The d120-induced cell death is partially mediated by cathepsin B, L and S**

Because the activities of cathepsins B and H were increased notably in d120-infected U937 cells, we next studied whether cathepsins mediate cell death in d120-infected U937 cells. The proportion of viable cells was not increased in d120-infected U937 cells treated with cathepsin H or L inhibitors (Fig. 4), even though the inhibitor treatment decreased the cathepsin H and L activities significantly (data not shown). In contrast, the proportion of viable cells was increased significantly in d120-infected cells treated with cathepsin B or S inhibitors (P=0.024 and P=0.023, respectively) (Fig. 4). Since d120-induced cell death was mainly apoptotic, we also studied the effects of cathepsin inhibitors on the number of apoptotic cells in d120-infected U937 cells. Cathepsin L inhibitor decreased the number of apoptotic cells significantly when compared with untreated d120-infected cells (P=0.007) (Fig. 4).

**d120 induces caspase 3 activation in U937 cells**

Since cathepsins can trigger caspase-dependent cell death, we studied whether increased cathepsin activity resulted in increased caspase 3 activity in d120-infected U937 cells. Caspase 3 activity was increased remarkably at a late time point of infection in d120-infected cells when compared with uninfected cells or parental virus HSV-1 (KOS)-infected cells (P<0.001 and P<0.001, respectively), and in R7041-infected cells when compared with uninfected cells (P<0.001) (Fig. 5). There was no significant difference in caspase 3 activity between d120-infected or R7041-infected and uninfected U937 cells at an early time point of infection (data not shown). Next, since caspase 3 was clearly activated in d120-infected U937 cells, we wanted to study whether caspase 3-specific inhibitor would decrease the amount of d120-induced apoptosis in U937 cells. Treatment with caspase 3 inhibitor blocked the activity of caspase 3 significantly, but no decrease in the amount of d120-induced apoptosis was seen (not shown).
Cathepsin L contributes to caspase 3 activity in U937 cells

To study the roles of cathepsins in d120-induced caspase 3 activation further, d120-infected cells were treated with cathepsin inhibitors and studied for caspase 3 activity. Inhibition of cathepsin L activity reduced caspase 3 activity in d120-infected cells significantly when compared with untreated d120-infected cells ($P = 0.019$) (Fig. 6). In contrast, inhibition of cathepsins B, H or S did not alter caspase 3 activity when compared with untreated d120-infected cells (Fig. 6).

DISCUSSION

The purpose of this paper was to characterize the HSV-1 ICP4 and Us3 deletion mutant d120-induced cell death in monocytic cells. In this paper, we have shown that d120 virus infection caused massive cell death in U937 cells. The increased amount of cell death in d120-infected cells was because of apoptosis mainly, but was also, in part, because of necroptosis. To our knowledge, this is the first report on necroptosis in HSV-infected cells. Necroptosis, or programmed necrosis, was recently described in caspase-independent cell death, which is regulated partly by the same mechanisms as apoptosis (Declercq et al., 2009; Degterev et al., 2005; Tait & Green, 2008). So far, little is known about necroptosis in virus infections but vIRA has been shown to interfere with necroptosis in murine cytomegalovirus-infected cells (Upton et al., 2010).

Since cathepsins can mediate apoptosis and necroptosis (Conus & Simon, 2008; Zhang et al., 2009), we studied the roles of cathepsins in d120-induced cell death. Lysosomal membrane permeabilization was increased significantly in d120-infected cells. This finding suggested that lysosomal cathepsins were released into the cytosol during d120 infection. Finding increased cathepsin activities in d120-infected cells supported this hypothesis. Cathepsin B and H activities were increased notably in d120-infected monocytic cells when compared with uninfected cells.

To study the roles of cathepsins in d120-induced cell death, we used cathepsin-specific inhibitors. When cathepsin B or S activities were prevented by specific inhibitors, the number of viable cells was increased significantly in d120-infected cells when compared with untreated d120-infected cells. Moreover, when cathepsin L activity was decreased by cathepsin L inhibitor the number of apoptotic cells was decreased significantly. These results suggest that cathepsins...
B and S mediate non-apoptotic cell death and that cathepsin L is involved in apoptosis in U937 cells. Although we could not detect a significant increase in the activity of cathepsin L in d120-infected cells the changes may have been masked by variation. Since cathepsin L is rather unstable at the neutral pH of the cytosol, changes in the actual cathepsin L activity could be difficult to measure using this assay. Moreover, the substrate used for the cathepsin L assay was also the substrate for cathepsin B; even if cathepsin B activity was blocked with the cathepsin B inhibitor, the detected cathepsin activity could be the sum of the activities of cathepsins L and B. However, since cathepsin L-inhibitor treatment decreased the amount of apoptosis in d120-infected cells, it appears that cathepsin L is active in d120-infected cells.

Since cathepsin L is known to mediate caspase 3-dependent apoptosis (Hsu et al., 2009), we studied caspase 3 activity in monotypic cells. The HSV-1 protein kinase Us3 blocks caspase 3-mediated apoptosis (Benetti & Roizman, 2007; Hagglund et al., 2002). Thus the HSV-1 ICP4 and Us3 deletion mutant virus is not able to block caspase 3 activation. As expected, caspase 3 activity was increased remarkably in d120-infected cells when compared with uninfected or parental-virus-infected cells. However, caspase 3 inhibitor treatment did not decrease the number of apoptotic cells in d120-infected U937 cells. In contrast, inhibition of cathepsin L activity reduced significantly both the number of apoptotic cells and caspase 3 activity in d120-infected cells. Taken together, these results may imply that, in d120-infected monotypic cells, cathepsin L activates caspase 3 and thus mediates d120-induced apoptosis. The contributions of cathepsin L and caspase 3 to d120-induced apoptosis remain to be elucidated further. The mechanism(s) controlling lysosomal membrane permeabilization or the hierarchy within the apoptosis signalling cascade is not yet fully understood. Whether lysosomal membrane permeabilization and cathepsin release into the cytosol are either initiating or amplifying events in d120-induced cell death should be explored. According to a study published recently, lysosomal membrane permeabilization and cathepsin release are caspase 3- and 7-mediated late events and thus are amplifying events for apoptosis (Oberle et al., 2010). Currently, lysosomal membrane permeabilization has been reported to be involved in HIV-1- and parvovirus H1-induced apoptosis (Di Piazza et al., 2007; Laforge et al., 2007). In addition, the involvement of autophagy in d120 infection cannot be excluded.

**METHODS**

**Cells.** Human monotypic cells (U937; ATCC) were cultured in RPMI 1640 medium (Gibco) supplemented with 10% FCS and 50 µg gentamicin ml⁻¹. Cell cultures were maintained at 37 °C in 5% CO₂. For cell viability, and cathepsin and caspase activity assays, 60 000 cells were plated on 96-well Isoplate black culture plates (PerkinElmer).

**Viruses.** Wild-type HSV-1 (F) and HSV-1 (KOS), Us3 deletion virus derived from HSV-1 (F) (R7041) (Purves et al., 1987) and its rescue virus (R7306) (Purves et al., 1987), and the Us3 and ICP4 deletion virus derived from HSV-1 (KOS) (d120) (DeLuca et al., 1985) were used for HSV-1 infection. HSV-1 stocks with high infectivity titres (>5 × 10⁶) were propagated in Vero cells or ICP4-transfected Vero E5 cells (d120). Cells were infected with HSV-1 at an m.o.i. of 5 and the infections proceeded at 37 °C in 5% CO₂. R7041 and R7306 viruses were generously provided by Dr B. Roizman (University of Chicago), d120 virus and E5 Vero cells were kind gift from Dr N. DeLuca (University of Pittsburgh) and HSV-1 (KOS) was kindly provided by Dr W. F. Goins (University of Pittsburgh).

**Chemical reagents.** Caspase 3 inhibitor (Z-DEVD-FMK; R&D Systems), cathepsin B inhibitor (CA-074Me; Bachem), cathepsin H inhibitor (H-Leu-CMK; Bachem), cathepsin L inhibitor [Z-Phe-Tyr(t-Bu)-DMK; Bachem], cathepsin S inhibitor (Z-Val-Val-Nle-DMK; Bachem) and Nec-1 (Tocris Bioscience) were diluted in DMSO. U937 cells were incubated with inhibitors (all at 10 µM except for Nec-1, which was at 50 µM) or 0.1% DMSO alone for 3 h prior to HSV-1 infection. Cathepsin B substrate (Z-Arg-Arg-AMC; Bachem), cathepsin H substrate (Z-Arg-Arg-AMC; Bachem), cathepsin B substrate (Z-Arg-Arg-AMC; Bachem), cathepsin S substrate (Z-Val-Val-Arg-AMC; Bachem) were diluted in DMSO. In caspase L activity assays, caspase B inhibitor was used to block the caspase B activity.

**Cell death.** The viability of the cells was studied by using the CellTiter-Glo luminescent cell viability assay kit (Promega) according to the manufacturer’s instructions. To study the proportion of necrotic cells, the cells were treated with 50 µM Nec-1 beginning 3 h before infection. Cell viability was measured at early (5 h) and late (21 h) time points of infection for four parallel samples. The number of apoptotic cells was determined using early apoptosis marker Annexin-V-Fluos (Caltag). For apoptosis analysis, 10 000 cells were collected with a FACScan flow cytometer (BD Biosciences) and analysed with Cell Quest software (BD Biosciences).

**Lysosomal membrane stability assay.** U937 cells (1 × 10⁵) were infected with HSV-1 and stained at a late time point of infection with acridine orange (Invitrogen) as described by Tokano et al. (1997) and Zhao et al. (2001). The fluorescence of the cells was studied with a FACScan flow cytometer. For analysis, 10 000 cells were collected and analysed with Cell Quest software. Reduced red fluorescence correlated with the reduced number of intact lysosomes.

**Cathepsin activity.** Cathepsin activity was studied as described previously (Peri et al., 2007). Briefly, U937 cells were washed with serum-free RPMI 1640 medium and incubated with 0.1 ml Hank’s balanced salt solution at 37 °C for 30 min. After 30 min incubation, the buffer was replaced with fresh buffer containing either 100 µM cathepsin B, 50 µM cathepsin H, 100 µM cathepsin L or 25 µM cathepsin S, fluorophore-conjugated substrate and 0.1% Triton X-100 to liberate the total cellular cathepsin activity. A Victor 1420 multilabel counter (PerkinElmer) was used to monitor fluorescent product formation (355 nm/460 nm) at 37 °C for 20 min.

**Caspase activity.** To measure caspase 3 activity, U937 cells grown on a 96-well plate were washed with PBS (pH 7.4) and incubated with 50 µl of lysis buffer, containing 10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA and 0.1% Triton X-100, on ice for 30 min. After cell lysis, 50 µM caspase 3 substrate (Z-DEVD)2-rhodamine 110 (Bachem) in 50 µl of reaction buffer, containing 20 mM Pipes, pH 7.4, 4 mM EDTA, 0.2% CHAPS and 10 mM DTT, was added onto the cells and the cells were incubated at 37 °C for 30 min. The plate was covered and incubated at 37 °C for 30 min. The progress of fluorescent product formation (485 nm/535 nm) was monitored for 80 min with a Victor 1420 multilabel counter.
Statistical analyses. For statistical analyses Student’s t-test was used. Values from HSV-1-infected cells were compared with uninfected cells or with untreated cells. Values of $P<0.05$ were considered statistically significant.

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