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Nitric oxide production induced by herpes simplex virus type 1 does not alter the course of the infection in human monocytic cells

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Undifferentiated U937 cells were not susceptible to herpes simplex virus type 1 (HSV-1) infection, but after differentiation with phorbol 12-myristate 13-acetate an increase in the permissivity to the virus was observed accompanied by the production of significant levels of viral particles. High levels of nitric oxide (NO) were produced in differentiated U937 cells infected with HSV-1. This production was comparable to that observed after addition of the NO donor glycerine trinitrate. The levels of NO drastically decreased when the cells were incubated with L-monomethyl arginine (L-NMA), an inhibitor of NO synthase. Although similar levels of NO were sufficient to decrease susceptibility of U937 cells to other viruses, neither incubation with NO donors nor addition of L-NMA altered the permissiveness to HSV-1 infection. Thus, these results suggest that NO does not interfere with the replication of HSV-1 in U937 cells.

Sensory neurons have been identified as the main site of latent infection by herpes simplex virus type 1 (HSV-1) (Halford *et al.*, 1996), but little is known about the mechanisms of persistence and reactivation in this cell type. Both HSV-1 and HSV-2 can also infect and establish persistent infections in cells of the immune system (Braun *et al.*, 1984). Freshly isolated T cells are nonpermissive for virus replication. Nevertheless, after mitogen stimulation, replication of HSV-1 is supported (Hammer *et al.*, 1982). In addition to neurons and T lymphocytes, human monocytes and macrophages can also be infected with HSV-1. Similar to T cells, freshly isolated or non-activated monocytic cells are refractory to virus production (Albers *et al.*, 1989). However, after triggering differentiation to macrophage-like cells, the capacity to produce infectious virus increases (Albers *et al.*, 1989; Tenneey & Morahan, 1991). This process of persistent infection might represent an important mechanism for virus dissemination during infection, as described for this and other viruses (Peluso *et al.*, 1985; López-Guerrero *et al.*, 1990; Valentin *et al.*, 1990).

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U937 cells have been widely used to study the infection of human monocyte-like cells with a number of animal viruses, including poliovirus (López-Guerrero *et al.*, 1989, 1990), dengue virus (Peiris & Porterfield, 1979), parvovirus (López-Guerrero *et al.*, 1997), human immunodeficiency virus (HIV) (Hammer *et al.*, 1986) and HSV (Tenney & Morahan, 1987, 1991; Stewart *et al.*, 1992). The infection of U937 cells by HSV-1 leads to persistence of the virus and undetectable levels of HSV-1 DNA replication. A marked dysfunction in the activity of the viral immediate-early transactivating protein ICP0 is a possible contributory factor (Stewart *et al.*, 1992). When U937 cells are treated with an inducer of differentiation such as phorbol 12-myristate 13-acetate (PMA), mezercin or vitamin D3, cells mature to a state permissive for HSV-1 replication, but the mechanisms underlying this process are still unknown. Agents that block the production of nitric oxide (NO) induced by IFN-γ treatment increase the susceptibility of murine macrophages to HSV-1 infection (Croen, 1993) and, in addition, NO donors protect against '*in vitro*' infection. However, no induction of NO has been detected during HSV-1 infection so far.

In the present report, we have studied both the effect of HSV-1 infection on the differentiation process of the human promonocytic cell line U937, and the effect of the production of NO during infection.

The establishment of cytopathic effects (CPE) by HSV-1 on U937 cells treated with differentiation inducers has been already described (Tenney & Morahan, 1987, 1991; Stewart *et al.*, 1992). However, the molecular mechanisms underlying the shift in the permissiveness of U937 cells upon induction of differentiation remain undefined. U937 cells were cultured in RPMI 1640 (Life Technologies) supplemented with 10% heat-inactivated foetal calf serum in a 5% CO_2 atmosphere at 37 °C. Untreated cells were resistant to HSV-1 (Kos strain) infection and no significant morphological changes were detected. In agreement with previous reports, after commitment to a more differentiated state induced by treatment with 50 ng/ml PMA

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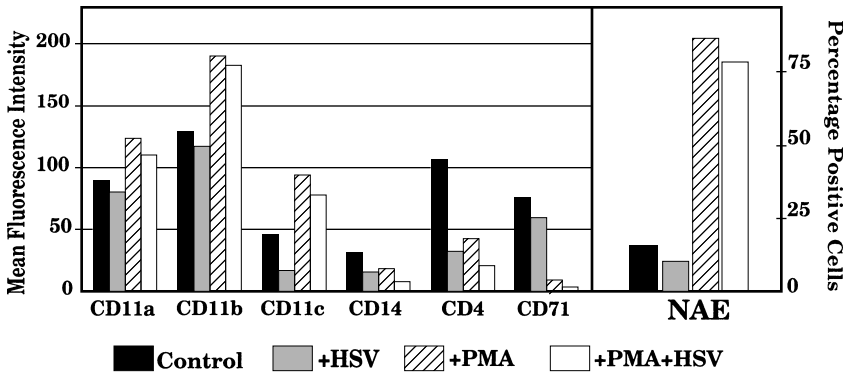

Fig. 1. Effect of HSV-1 infection on the maturation of U937 cells. Parallel cultures of U937 cells, either untreated or treated with 50 ng/ml PMA, were mock-infected or infected with HSV-1 (10 p.f.u. per cell). After incubation for 24 h at 37 °C, cells were subjected either to fluorescence flow cytometry analysis with the indicated monoclonal antibodies (left) or to measurement of α-naphthyl acetate esterase activity (right). Results shown are average values from three independent experiments (SD < 22%).

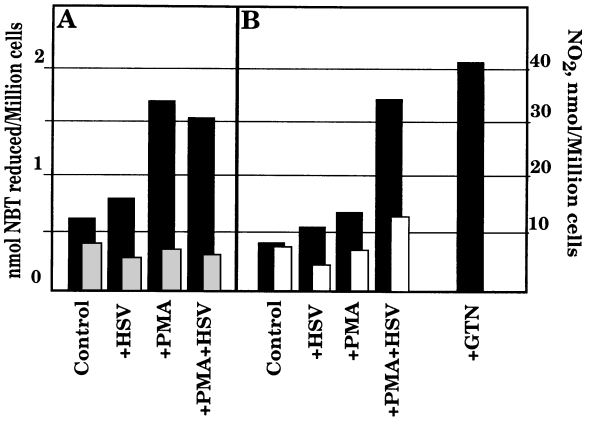

Fig. 2. Effect of HSV-1 infection on the activation steady-state of immature and differentiated U937 cells. U937 cells, either untreated or treated with 50 ng/ml PMA for 48 h, were mock-infected or infected with HSV-1 (10 p.f.u. per cell) in the presence of 500 IU/ml of SOD (grey bars), 2 mM L-NMA (white bars) or in the absence of these compounds (black bars). After incubation for 24 h at 37 °C, O_2^- (A) and NO (B) production were quantified. Average values from triplicate cultures are shown (SD < 25%).

(Sigma), cells became more susceptible to the HSV-1 triggered CPE (data not shown).

The phenotypic changes associated with maturation of the promonocytic cell line U937 and its susceptibility to virus infection have been previously examined using a variety of virus systems (López-Guerrero *et al.*, 1989, 1990, 1997). Studies in poliovirus-infected U937 cells (López-Guerrero *et al.*, 1989) showed an interference of the virus with the patterns of gene transcription and expression of differentiation-specific surface antigens induced by PMA. In the case of U937 infection by the autonomous parvovirus H-1, the role of the modulation of certain antigens observed after infection has not been unravelled (López-Guerrero *et al.*, 1997). To analyse the effects of HSV-1 infection, 5×10^5 untreated or PMA-treated U937 cells were examined by fluorescence cytometry, 24 h after virus addition, using a panel of monoclonal antibodies directed to different cell surface markers as described by López-Guerrero *et al.* (1997). Fig. 1 shows that in normal U937 cells, HSV-1 infection significantly lowered the surface expression of CD11c, CD14 and CD4. After induction of differentiation by PMA treatment, the expression of these antigens in HSV-1-infected cells closely paralleled that observed in mock-infected cells. The expression of non-specific esterases, a differentiation-specific marker, determined with a α-naphthyl acetate esterase (NAE) kit (Sigma), did not show modification upon HSV-1 infection.

To analyse the effect of HSV-1 infection on the activation state of U937 cells, we measured the production of both superoxide anion (O_2^-) and NO. Superoxide anion and NO were analysed by quantifying the extent of blue tetrazolium (NBT; Sigma) reduction at 620–450 nm and the production of NO_2 at 550 nm, respectively, as described by López-Guerrero *et al.* (1997). Fig. 2(A) shows that untreated U937 cells produced basal levels of O_2^-. Incubation with PMA for 48 h increased the levels of O_2^- significantly. However, in contrast to the results observed with poliovirus (López-Guerrero *et al.*, 1991) and parvovirus (López-Guerrero *et al.*, 1997), HSV-1 infection did not affect this increase. Nevertheless, a notable increase in NO production was detected in PMA-differentiated U937 cells infected with HSV-1 but not in uninfected PMA-treated cells (Fig. 2B). The levels of NO were comparable to that obtained upon addition of the NO donor glycerol trinitrate (GTN) (Merck), used at a final concentration of 6 mg/ml. Controls to show the specificity of O_2^- and NO production included assays in the presence of the O_2^- production inhibitor human superoxide dismutase (SOD) (Sigma) or the arginine analogue N^G-monomethyl-L-arginine (L-NMA) (Calbiochem-Novabiochem), respectively (Fig. 2).

The fact that the production of NO by PMA-treated U937 cells infected with HSV-1 correlated with increased susceptibility to the virus was unexpected. Previous studies (Croen, 1993) showed that the induction of NO by IFN-γ resulted in a 1000-fold inhibition of HSV-1 replication in a murine macrophage cell line. Thus, the possible role of the production of NO in the susceptibility of U937 cells to HSV-1 was further studied. U937 cells (2×10^5), either undifferentiated or differentiated by treatment with 50 ng/ml PMA for 24 h, were infected at 10 p.f.u. per cell in the absence or presence of 2 mM L-NMA or 6 mg/ml GTN. The viability of the cells and the production of infectious particles were

Table 1. Effect of NO production on the susceptibility of U937 to HSV-1 infection

Pretreatment	Virus production (p.f.u. per cell)*	Cell viability†
None	0·11	87
PMA	3·8	39
L-NMA	0·21	82
GTN	0·16	79
PMA + L-NMA	4·4	47
PMA + GTN	4·2	43

* Virus titrations were performed by plaque-assay on HeLa cell monolayers.
† Survival of HSV-1-infected cells was determined by the trypan blue exclusion technique and the values obtained are expressed as a percentage relative to the survival of mock-infected cultures (SD $<$ 25 %).

determined 48 h after infection by plaque assay on HeLa cell monolayers. Table 1 shows a clear increase in infectious virus production and a decrease in the viability of infected PMA-treated cells compared to untreated cells infected with HSV-1. However, neither inhibition of NO synthase nor incubation with the NO donor modified the course of infection.

The role if any of NO production in antiviral mechanisms is still controversial (Croen, 1993; Karupiah *et al.*, 1993; Akaike *et al.*, 1996; Tucker *et al.*, 1996; Lowenstein *et al.*, 1996; Mikami *et al.*, 1996; Kreil & Eibl, 1996; Guillemard *et al.*, 1996). Croen (1993) demonstrated that the addition of exogenous NO inhibited the infection of a murine cell line by HSV-1. However, even in this case, no endogenous NO production was detected after infection. We have recently described the possible role of production of NO and O_2^- in the resistance of U937 to parvovirus H-1 infection (López-Guerrero *et al.*, 1997). However, those studies were performed using constitutively activated U937 cells resistant to the parvovirus. The present study shows that NO production is induced in differentiated human monocytic cells after HSV-1 infection, although no effect of NO on the course of the infection was observed. It has been suggested that exogenous NO might have effects different from endogenously produced NO. Thus, NO donors can inhibit HIV-1 replication in human peripheral blood mononuclear cells, but HIV-1 replication can take place in the presence of endogenously produced NO, and this NO might be mediating some of the neurotoxic effects induced by the virus (Bukrinsky *et al.*, 1995). Production of NO by inflammatory cells in a murine model of coxsackievirus B3-induced viral myocarditis has also been reported (Mikami *et al.*, 1996), but the authors did not discriminate whether NO plays cytotoxic or cytoprotective roles in the pathogenic mechanisms of myocardial dysfunction. Using infected mice fed with NO synthase inhibitors, Lowenstein *et al.* (1996) obtained increased titres of coxsackievirus and a higher percentage mortality than in control infected mice, and postulated that NO inhibited the replication of this virus in murine myocarditis. On the other hand, Kreil & Eibl (1996) reported that murine macrophages infected with tick-borne encephalitis virus, a flavivirus, were not affected by induction of NO, and it was suggested that NO might contribute to the pathogenesis observed in infected animals.

It can be argued that the observed production of NO upon HSV-1 infection of PMA-treated U937 cells represents an event which makes sense only in a whole animal '*in vivo*' context, in which stimulation of specific immune responses takes place, and that it does not play a significant role in '*in vitro*' infected cells. This view is shared by other authors (Guillemard *et al.*, 1996; Tucker *et al.*, 1996). Thus, it has been observed that exogenous NO generation causes a dose-dependent inhibition of encephalomyocarditis virus (EMCV) growth in murine L-929 cells. These cells can spontaneously produce endogenous NO, but this is not modified by EMCV infection and does not exert any antiviral activity (Guillemard *et al.*, 1996). Similarly, NO protects mice from fatal encephalitis by a mechanism that does not directly involve the inhibition of Sindbis virus replication (Tucker *et al.*, 1996). Rather, in this example, NO might be protecting infected neurons until the specific immune response can control the infection.

Epstein–Barr virus (EBV) latency is maintained by NO through down-regulation of the expression of the immediate-early EBV transactivator Zta (Mannick *et al.*, 1994), preventing the apoptosis mechanism induced by viral infection. Therefore, the effect of HSV-1 on induction of apoptosis in U937 cells was investigated. However, after infection of PMA-treated or untreated U937 cells, no evidence of specific degradation of the cellular genomic DNA was detected. Furthermore, no effect on induction of apoptosis was observed in the presence of L-NMA (data not shown), suggesting that in contrast to EBV the persistence of HSV-1 in U937 cells and the increased cell death after infection of differentiated cells might not be due to modulation of apoptosis.

In summary, the susceptibility of monocytic U937 cells to infection with HSV-1 increased after commitment to cell differentiation. Modification of the production of NO was detected, and this represents the first indication of direct induction of NO by HSV-1 infection of human immune system cells. However, this alteration does not seem to play a role in the susceptibility of the host cells to the '*in vitro*' infection. The possibility remains that this alteration in the state of cellular activation might have a role when occurring in a more physiological context where interactions with other cells of the immune system can take place.

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