

# Induction of Apoptosis by Herpes Simplex Virus-1 in Neonatal, But Not Adult, Neutrophils

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**ABSTRACT:** We report a study on the effect of herpes simplex virus 1 (HSV-1) infection on apoptosis of neutrophils from both adults and neonates and present evidence showing that HSV-1 enhances apoptosis in neonatal, but not adult, neutrophils. HSV-1 enhanced the expression of both Fas and Fas ligand on the surface of neonatal neutrophils. Treatments with anti-Fas antibody and a Fas ligand inhibitor significantly reduced the induction of apoptosis by HSV-1. Using an ELISA assay, it was found that HSV-1 infection also leads to increased release of soluble FasL from HSV-1-infected neonatal neutrophils. Increased neonatal neutrophil apoptosis following HSV-1 infection may represent an important mechanism by which HSV-1 may diminish the antiviral response of neonatal neutrophils and might explain, at least in part, the severity of infections that are caused in newborns by this herpesvirus. (*Pediatr Res* 59: 7–12, 2006)

Host defense against viral infections involves both innate and specific immune responses. The effector cells in nonspecific immune response are mainly macrophages, neutrophils, and natural killer (NK) cells (1). The widespread presence of neutrophils suggests that they are among the initial leukocytes implicated in host defense against invading pathogens. The anti-infectious response of neutrophils involves the phagocytosis of microbial agents (2), the production of degradative enzymes and oxygen free-radicals, as well as the synthesis of immunoactivating proteins that initiate the mechanisms of the specific immune response (3,4). However, the effects of viral infections on neutrophils have not been well elucidated.

Neutrophils are terminally differentiated leukocytes, with a half-life of only 6–20 h, and they spontaneously undergo apoptosis (5). Apoptosis represents a mechanism of controlling the functional longevity of neutrophils in tissues and therefore affects their participation in the control of infections. Neonatal neutrophils have been demonstrated to have decreased chemotaxis and diminished phagocytotic abilities (6).

HSV-1 is the most common cause of fatal sporadic viral encephalitis in North America. For HSV infection with disseminated sepsis, mortality rates are 50–60%, with only 60% of survivors having a normal neurologic outcome after 1 y (1,7). Host responses in newborns with HSV infection are

different from in adults and are reflected by the increased morbidity and mortality associated with this infection in newborns (8). There are no data evaluating the effect of HSV-1 infection on neutrophil apoptosis. Because previous studies had indicated a developmental immaturity of cord blood neutrophils (9–12), we investigated the effects of HSV-1 infection on neutrophil apoptosis and examined whether neonatal and adult neutrophils may differ in this regard. As neutrophils break up into multiple apoptotic bodies that contain live viruses, they will facilitate the spread of viruses as the apoptotic bodies are engulfed by macrophages and bypass the immune system (13). Innate immunity as manifested by NK cell activity plays an important role in HSV infection via monocyte activation and IL-15 secretion (14). Therefore, neutrophil apoptosis may alter immune activation of the humoral immune system by decreasing neutrophil cytokine secretion. HSV-1-infected, neutrophil-depleted mice became progressively cachectic and developed signs of encephalitis, with brain virus titers being significantly higher compared with control animals (15).

Fas is expressed on neutrophils' plasma membrane and when it binds to FasL, which is constitutively expressed on neutrophils; this cross-linking leads to apoptosis (5,16). Neutrophils also release sFasL (5), thus providing a paracrine pathway for the neutrophils to mediate their own programmed cell death. We therefore examined the expression of Fas and FasL by HSV-1-infected neutrophils and measured the release of sFasL. We present evidence showing that neonatal, but not adult, neutrophils undergo apoptosis in response to an *in vitro* infection with HSV-1 in a Fas/FasL-dependent interaction.

## MATERIALS AND METHODS

**Neutrophil isolation.** Umbilical venous blood samples were obtained from the fetal side of placentas of healthy, full-term neonates within 5 min of birth in the obstetrical unit of Sainte-Justine Hospital, and venous blood was collected from healthy adult volunteers. The Institutional Ethics Review Board at Sainte Justine Hospital approved the study. Neutrophils were isolated using two gradients of Percoll (Sigma Chemical Co., St. Louis, MO), 62% and 76% (17). The neutrophils were washed three times with PBS (pH 7.2) and resuspended in Iscove's modified Dulbecco's medium IMDM

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**Abbreviations:** FasL, Fas ligand; HSV-1, herpes simplex virus 1; MCF, mean channel fluorescence; PI, propidium iodide; PMN, polymorphonuclear neutrophils; sFasL, soluble Fas ligand

(Sigma Chemical Co.) with 1% of heat-inactivated autologous plasma. The purity of all neutrophil preparations used was evaluated by cell analyzer (Technicon H3 RTX, Bayer Diagnostics, Tarrytown, NY) that measured myeloperoxidase and was found to be >95%.

**Virus preparation and neutrophil infection.** HSV-1 was produced in vero cells as previously described (18). Vero cells were infected by HSV-1 and incubated at 37°C until the cytopathic effect in infected cell monolayer reached 70%. Cells were harvested and centrifuged. Cell-free culture supernatants were prepared and the pellet was frozen and thawed three times, centrifuged for 10 min at 2000 rpm, and the supernatant was added to the first one. All the supernatants were filtered through a 0.45- $\mu$ m pore size filter. Viral particles were harvested by differential centrifugation. Virus stocks were resuspended in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) aliquoted, and stored at -80°C. Viral titer was measured by cytopathic effect using the Reed-Muench method (19); the titer was expressed as the 50% tissue culture infectious dose (TCID<sub>50</sub>) and was determined by evaluating the number of vero cells exhibiting cytopathic effect (CPE). The virus stock had a titer of 10<sup>9</sup> 50% tissue culture infective dose/mL (10<sup>9</sup> TCID<sub>50</sub>). The mock control was prepared from uninfected vero culture supernatant. Both virus and mock control stocks were aliquoted and stored at -80°C.

For all the experiments, 10<sup>6</sup> neonatal and adult neutrophils were incubated in the absence or presence of HSV-1 at a multiplicity of infection of 25 TCID<sub>50</sub> per cell in a 5-mL polystyrene round-bottom tube (Falcon 352058, Becton Dickinson Labware, Franklin Lakes, NJ) for 20 h at 37°C and 5% CO<sub>2</sub>. A 20-h incubation was used since the viral cycle of herpes simplex virus is 20 h and we wanted to evaluate the effect of a single viral multiplication on neutrophil apoptosis (1). The media and all reagents were certified endotoxin-free.

**Detection of HSV-1 protein in infected neutrophils.** Neutrophils were incubated in the presence or absence of HSV-1. The Cytofix/Cytoperm Plus Kit (BD PharMingen, San Diego, CA) treatment was used to permeabilize and fix neutrophils membrane. Control and HSV-1-infected neonatal and adult neutrophils were stained for 30 min at 4°C with FITC-conjugated MAB specific for HSV-1 cytoplasmic protein (Clone H62; Medicorp, Montreal, Quebec, Canada; 20  $\mu$ L/sample). Negative control staining was revealed using FITC-conjugated murine IgG1 (4  $\mu$ L). The staining was performed for 30 min at 4°C in the dark; the cells were then washed twice with Perm wash (Cytofix/Cytoperm plus Kit), resuspended in staining buffer, and fixed with 4% paraformaldehyde in PBS. Neutrophils were then analyzed by flow cytometry. Neutrophils were also examined by fluorescence microscopy for HSV-1 protein expression. After their incubation with or without HSV-1, the cells were washed three times with PBS, fixed with cold acetone/methanol, and then stained with the MAB to HSV-1 protein (Clone H62; Medicorp; 20  $\mu$ L/sample) for 1 h at 37°C. The cells were then washed with PBS four times, fixed, and examined by fluorescence microscopy.

**Analysis of apoptotic cells by flow cytometry.** After incubation, we identified normal and apoptotic cells based on their PI and annexin-V-FITC staining using a commercially available kit (Roche Molecular Biochemicals, Mannheim, Germany). After incubation, neutrophils were washed twice with PBS, then resuspended in 100  $\mu$ L of staining solution (prediluted 2  $\mu$ L annexin-V-fluorescein labeling reagent in 100  $\mu$ L HEPES buffer and adding 2  $\mu$ L PI) and incubated for 10–15 min at 15–20°C. Ten thousand neutrophils were then analyzed by flow cytometry (FACScan, BD Biosciences, Oakville, ON, Canada). The neutrophils were gated using forward scatter and side scatter to only consider a homogenous population of neutrophils within our samples. Viable cells were defined as annexin-V negative and PI negative, and apoptotic cells were defined as annexin-V positive and PI negative. Annexin-V binds exposed phosphatidylserine molecules on the apoptotic cell membrane.

**Cell surface expression of Fas and FasL by HSV-1-infected neutrophils.** Cell surface expression of Fas and FasL by HSV-1-infected neutrophils was determined by flow cytometry using 20  $\mu$ L of murine anti-human Fas MAB (IgG1, clone UB2; MBL, Naka-ku, Nagoya, Japan) labeled with FITC and 4  $\mu$ L of anti-human FasL NOK-1 for primary staining (BD PharMingen, Mississauga, ON, Canada). The expression was revealed using 4  $\mu$ L of FITC-conjugated purified goat anti-mouse IgG (BD Biosciences, San Jose, CA) as secondary antibody. Negative control staining was revealed using 4  $\mu$ L of murine IgG-1 (BD PharMingen). After incubation, the cells were washed twice with PBS and treated with specific antibodies for 45 min at 4°C, washed twice with PBS containing 0.1% sodium azide, and fixed with 4% paraformaldehyde in PBS before flow cytometry analysis of 10,000 cells per sample.

**Release of soluble form of FasL by neutrophils.** Cell-free supernatants from untreated and HSV-1-infected neutrophils were obtained after infection and tested in duplicate for the release of sFasL using an ELISA kit (MBL,

Naka-ku). The assay uses anti-FasL MAB against two different epitopes. Cell-free supernatants (100  $\mu$ L/sample) were used to quantify the released sFasL. The sensitivity of the assay is 0.1 ng/mL. The OD of each well was measured at 450 nm using a microplate reader (Tecan Spectra, Zurich, Switzerland). The concentration of sFasL was established from a dose-response curve using reference standards.

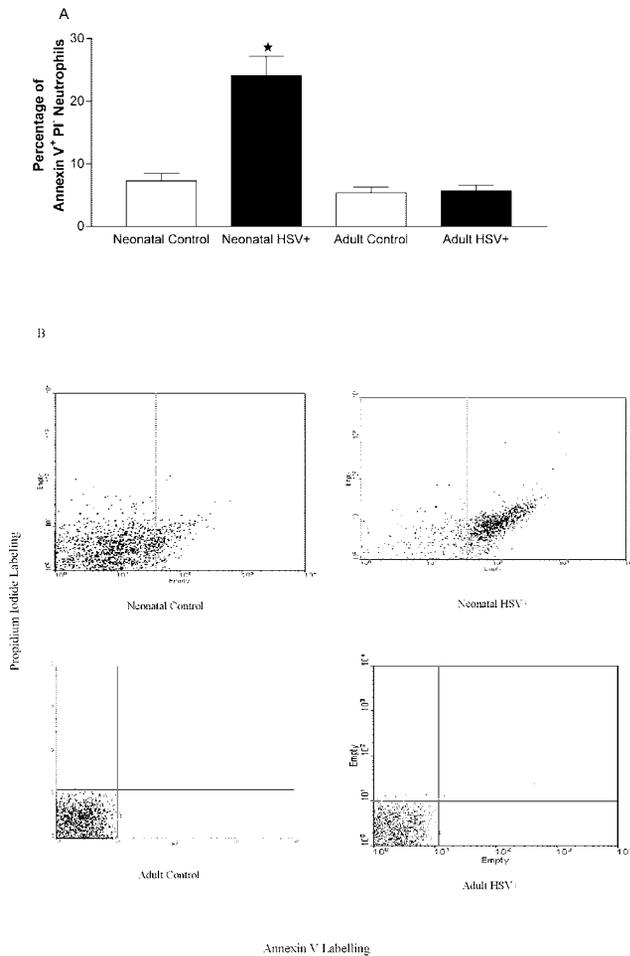
**Inhibition of apoptosis induction using anti-Fas antibody and FasL inhibitor.** Neutrophils (5  $\times$  10<sup>5</sup> cells/sample) were incubated in the absence or presence of HSV-1, and co-treated with anti-human-Fas MAB (BMS 140; Bender MedSystems, Medicorp, Montreal, Quebec, Canada; 5  $\mu$ g/mL) and FasL inhibitor (AF-016; Kamiya Biomedical Co., Seattle, WA; 4  $\mu$ g/mL). The concentration of anti-human-Fas and FasL inhibitor were determined as the minimal concentration that inhibited Fas-mediated apoptosis. Cells were then assessed for apoptosis using annexin-V and PI staining as described above.

**Statistical analysis.** Statistical analysis was performed using GraphPad InStat and Prism (GraphPad Software, San Diego, CA). The difference between HSV-1-infected and uninfected control neutrophils was evaluated using a paired *t* test. *p* Values < 0.05 were statistically significant.

## RESULTS

**HSV-1 induces apoptosis in neonatal neutrophils.** Twenty hours postinfection, HSV-1 increased the percentage of apoptosis in infected neonatal neutrophils compared with control uninfected as well as mock-infected neonatal neutrophils: 24  $\pm$  8.6% versus 7.2  $\pm$  3.3%, respectively (*n* = 8, *p* < 0.005; Fig. 1, A and B). In adults, HSV-1-infected neutrophils did not show such an increase in the percentage of apoptosis compared with control-uninfected neutrophils (5.7  $\pm$  2.2% versus 5.4  $\pm$  2.4%, *n* = 8; Fig. 1, A and B). Preincubation of HSV-1-infected neonatal neutrophils with 3  $\mu$ g/mL of acyclovir prevented the increase in neonatal neutrophil apoptosis. This concentration inhibits 50% of the herpes simplex virus growth *in vitro* (20). Treatment of neonatal neutrophils with inactivated virus did not increase apoptosis in these cells compared with neutrophils treated with infectious HSV-1. In addition, 6 h after HSV-1 infection, neonatal neutrophils showed no increase in their rate of apoptosis compared with controls (6.8  $\pm$  3.3% versus 4.9  $\pm$  2.8%). Furthermore, infection of neonatal neutrophils by HSV-1 at low infectious titre (*i.e.* 10<sup>3</sup> TCID<sub>50</sub>/mL) evaluated at 20 h postinfection did not induce neutrophil apoptosis compared with neutrophils treated with HSV-1 at high titre (10<sup>9</sup> TCID<sub>50</sub>/mL; 3.0  $\pm$  0.1% versus 24.1  $\pm$  8.6%, *p* < 0.001).

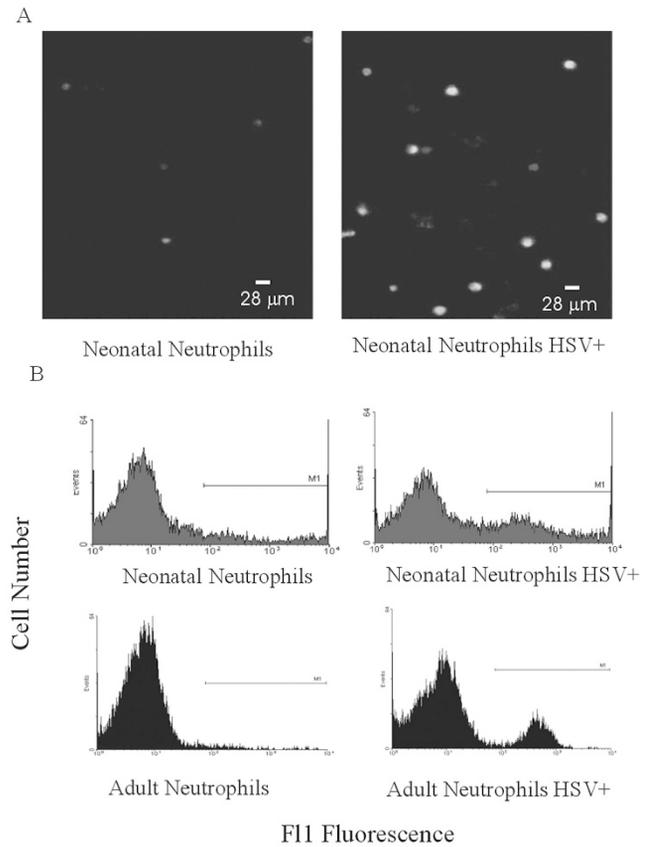
**HSV-1 infection of neutrophils.** We then determined whether HSV-1 replicates in the neutrophils by using HSV-1-specific antibodies and immunofluorescence assay. We found that expression of HSV-1 cytoplasmic protein in neutrophils indicated that viral proteins were present in these cells (Fig. 2 A). In HSV-1-infected neutrophils there was marked expression of cell staining for the HSV-1 protein. We also analyzed the presence of HSV-1 protein in infected cells compared with control cells by flow cytometry (Fig. 2B). There were 8  $\pm$  3% of infected neonatal neutrophils that stained for HSV-1 protein as opposed to the background staining of 3  $\pm$  1% in the control neutrophils (*p* < 0.0007). Similar evidence of neutrophil infection was demonstrated in adult neutrophils (10  $\pm$  4.4% versus 5.7  $\pm$  3.7%, *p* < 0.0007; Fig. 2B). The presence of HSV-1 staining in the uninfected cells is consistent with nonspecific staining as it was similar to IgG isotype control staining.



**Figure 1.** Effect of HSV-1 on neutrophil apoptosis. Representative profiles of flow cytometric analysis of HSV-1-infected neutrophils from a newborn and adult. (A) This graphical representation of the percentage of apoptotic neutrophils demonstrates a significant increase in apoptotic neonatal neutrophils after HSV-1 infection as opposed to control neutrophils ( $24 \pm 9\%$  vs  $7 \pm 3\%$ ;  $n = 8$ ;  $p < 0.005$ ). There is no increase in the percentage of apoptotic adult neutrophils between HSV-1-infected and control neutrophils ( $6 \pm 2\%$  vs  $5 \pm 2\%$ ;  $n = 8$ ). Data are presented as the mean  $\pm$  SD of each group. (B) There is an increase in the number of annexin-V-positive PI negative neutrophils (lower right quadrant) in the HSV-positive neonatal neutrophils as opposed to controls or adult neutrophils.

**Fas and FasL expression increases in HSV-1-infected neonatal neutrophils.** In neonatal neutrophils, HSV-1 infection led to an increase of MCF of Fas expression from  $64 \pm 13$  in uninfected neonatal neutrophils to  $176 \pm 28$  in infected neonatal neutrophils ( $p < 0.03$ ; Fig. 3, A and B). There was no significant difference in Fas expression between the infected and uninfected adult neutrophils (Fig. 3, A and C). In neonatal neutrophils, HSV-1 infection also led to an increase of FasL expression from  $234 \pm 53$  in controls to  $621 \pm 151$  in HSV-1-infected neonatal neutrophils ( $p < 0.05$ ; Fig. 4, A and B). In adult neutrophils, no significant difference in FasL expression was found between the infected and noninfected cells (Fig. 4, A and C).

**Fas and FasL inhibitors block apoptosis induced by HSV-1 in neonatal neutrophils.** To confirm the role of Fas and FasL, we treated the control uninfected cells and HSV-1-infected cells with inhibitors of Fas and FasL to see whether



**Figure 2.** HSV-1 infection of human neutrophils. (A) The presence of HSV protein in neutrophils was demonstrated using MAb to HSV-1 protein and detected by immunofluorescence. The staining seen in the uninfected neutrophils was consistent with nonspecific binding as it was identical to the IgG isotype control staining (photograph taken at  $400\times$  magnification). (B) The presence of HSV-1 intracellular protein in neutrophils was measured by flow cytometry. M1 represents the neutrophils containing HSV-1 protein. ( $8 \pm 3\%$  vs  $3 \pm 1\%$ ,  $p < 0.009$  in neonatal neutrophils and  $10 \pm 4.4\%$  vs  $5.7 \pm 3.7\%$  in adult neutrophils.)

there is a decrease in neutrophil apoptosis. When the HSV-1-infected neonatal neutrophils were co-incubated with blocking anti-Fas antibody, apoptosis decreased to control level ( $20\%$ ), i.e.  $23 \pm 2\%$  in the anti-Fas-treated and HSV-1-infected cells versus  $36 \pm 4\%$  in infected neutrophils ( $n = 5$ ,  $p < 0.01$ ) (Fig. 5). When the HSV-1-infected neonatal neutrophils were co-incubated with FasL inhibitor, the apoptosis decreased to the control level ( $14\%$ ), i.e.  $18 \pm 2\%$  in the FasL-inhibitor-treated and HSV-infected cells versus  $38.0\% \pm 4$  in infected neutrophils ( $n = 4$ ,  $p < 0.01$ ) (Fig. 5). Nonspecific IgG did not affect the rate of apoptosis of the neutrophils.

**Release of soluble FasL increases in neonatal neutrophils.** There was an increase in the release of sFasL, i.e.  $2600 \pm 552$  pg/mL from infected cells compared with  $352 \pm 475$  pg/mL from uninfected neonatal neutrophils (Fig. 6). In infected neutrophils, sFasL could induce apoptosis via linking to the Fas receptor in adjoining neutrophils. There was no increase in sFasL in infected adult neutrophils.

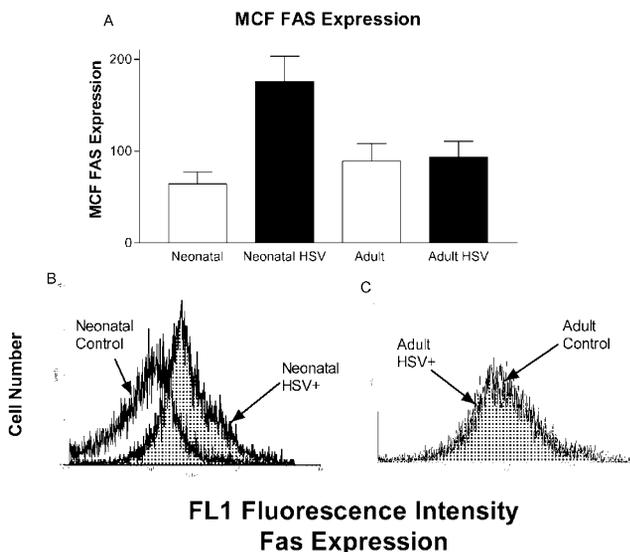
**DISCUSSION**

There are distinct differences between infants' and adults' immune responses. These variations are likely due to a com-

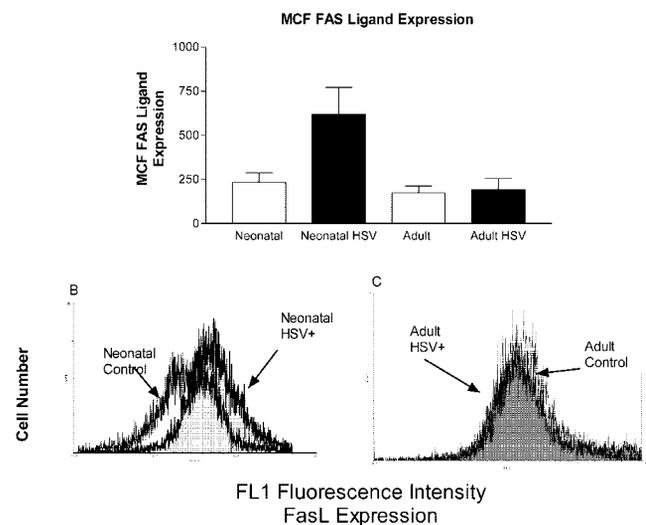
combination of factors, *e.g.* antibody deficiency, macrophages, NK cells, and T lymphocytes (1). Deficiency in neutrophil function appears to be a major host defense abnormality in the neonate (6,11,12,21). Apoptosis represents an efficient mechanism by which the virus can induce cell death and disseminate its progeny while limiting the induction of the inflammatory and immune responses. During apoptosis, the entire cellular contents, including progeny virions, are packaged into membrane-bound apoptotic bodies that are rapidly taken up by surrounding cells. This process severely limits the inflammatory response and allows the infection to spread undetected by the host organism (13).

There is a wide body of literature concerning HSV-1 and apoptosis. Modulation of apoptosis by HSV-1 during infection is dependent on virus strain and cell-type (22). Many viral proteins are involved in the inhibition or the induction of apoptosis. In epithelial cells the induction of apoptosis occurs between 3 and 6 h postinfection (23). After this period, early ( $\beta$ ) and late ( $\gamma$ ) proteins are synthesized and block apoptosis. Reports suggest that the  $\alpha$  protein ICP27, which stimulates and regulates the expression of the subsequent  $\beta$  and  $\gamma$  proteins (1), which block apoptosis in HSV-1-infected cells, is a central regulatory factor in the inhibition of apoptosis.

In the present study of the induction of apoptosis by HSV-1 in neonatal but not adult neutrophils, cell analysis at different postinfection time-points indicated that the percentage of apoptotic cells increased between 6 and 20 h postinfection. This would suggest that apoptosis of neonatal neutrophils depends on the expression of viral  $\beta$ - and/or  $\gamma$ -genes and maybe that adult neutrophils inhibit the expression of these genes. It has



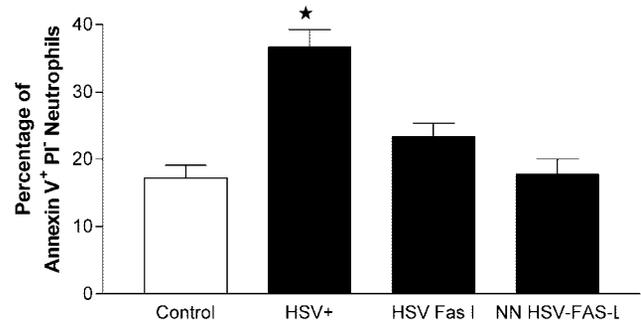
**Figure 3.** Effect of HSV-1 infection on Fas expression on neonatal and adult neutrophils. (A) There is a statistically significant increase in Fas MCF in neonatal neutrophils infected with HSV-1 compared with neonatal neutrophils and adult neutrophils MCF ( $176 \pm 28$  vs  $64 \pm 13$ ,  $89 \pm 19$ ,  $93 \pm 17$ ;  $n = 5$ ;  $p < 0.03$ ). There was no significant difference in Fas expression between the infected and noninfected adult neutrophils. (B) Cell surface expression of Fas in HSV-1-infected neonatal neutrophils. Fas expression was measured on HSV-1-infected neutrophils (dark area) compared with uninfected neutrophils (light area). (C) Cell surface expression of Fas in HSV-1-infected adult neutrophils. Fas expression was measured on HSV-1 infected neutrophils (dark area) compared with uninfected neutrophils (light area).



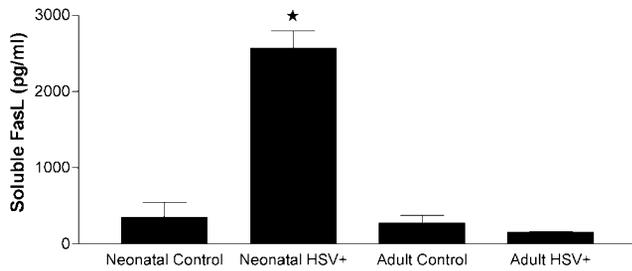
**Figure 4.** Effect of HSV-1 on FasL expression on neonatal neutrophils. (A) There is a statistically significant increase in FasL MCF in neonatal neutrophils infected with HSV compared with neonatal neutrophils and adult neutrophils MCF ( $620 \pm 151$  vs  $234 \pm 53$ ,  $174 \pm 40$ ,  $195 \pm 60$  ( $n = 5$ ,  $p < 0.05$ ). There was no significant difference in FasL expression between the infected and noninfected adult neutrophils. (B) FasL expression was measured on HSV-1 infected neutrophils (dark area) compared with control (light area). (C) Cell surface expression of FasL in HSV-1 infected adult neutrophils. FasL expression was measured on HSV-1 infected neutrophils (dark area) compared to uninfected neutrophils (light area).

been reported that pro-inflammatory cytokines such as tumor necrosis factor (TNF)- $\alpha$ , interferon (INF)- $\gamma$ , IL-1 $\beta$ , IL-2, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-15 can delay neutrophil apoptosis (24). However, some of these cytokines, INF- $\gamma$ , IL-12, and IL-18 (25) have been reported to have lower expression levels in neonatal neutrophils. This could explain why neonatal neutrophils are more susceptible to undergo apoptosis after HSV infection than adult neutrophils. However, this requires further investigation.

The rate of apoptosis in neonatal neutrophils may depend on several factors: 1) the infectious titre of the virus, *i.e.* the higher virus titre, the higher the increase of the apoptosis and



**Figure 5.** Fas and FasL inhibitors blocked the increase of apoptosis induced by HSV-1 in neonatal neutrophils. Cells treated with anti-Fas antibody reduced the HSV-1-infected cells to basic apoptosis level  $23 \pm 2\%$  in infected, anti-Fas-treated vs  $36 \pm 4\%$  in infected neutrophils ( $n = 5$ ,  $p < 0.01$ ). Cells treated with FasL inhibitor reduced the HSV-1-infected cells to basic apoptosis level  $18 \pm 2\%$  in infected, FasL-inhibitor-treated vs  $38 \pm 4\%$  in infected neutrophils ( $n = 4$ ,  $p < 0.01$ ). Data are presented as the mean  $\pm$  SD. \* $p < 0.01$  compared with control, Fas, and Fas blocker.



**Figure 6.** HSV-1 increased the release of soluble FasL by infected neonatal neutrophils *in vitro*—2600 ± 552 pg/mL in infected neutrophils vs 352 ± 475 pg/mL in uninfected neonatal neutrophils, 270 ± 290 pg/mL in uninfected adult neutrophils and 155 ± 24 pg/mL in infected adult neutrophils ( $n = 7$ ,  $p < 0.001$ ). Data are presented as the mean ± SD.

2) competition between noninfectious and infectious viral particles, *i.e.* infection of neutrophils by high- and low-titred virus or inactivated virus, did not induce the apoptosis in neonatal neutrophils. This suggests that noninfectious viral particles may compete with the infectious ones. In addition, to induce apoptosis, the virus should have minimum infectious dose (TCID<sub>50</sub> = 10<sup>7</sup>/mL), and should penetrate and replicate in neutrophils. UV-inactivated virus preparation did not induce apoptosis in neonatal neutrophils. Treatment of infected neutrophils with acyclovir also blocked the increased rate of apoptosis by HSV-1 infection. This suggests that viral replication was necessary to induce apoptosis. Heparan sulfate has been described as a major factor in binding of HSV-1 to the epithelial cell surface (26,27). Whether heparan sulfate also plays a major role in HSV infection of neutrophils is not known.

We may have expected that neonates who have HSV-1 infection might be susceptible to neutropenia. However, neutropenia is not a major clinical finding in neonates with HSV infection. Circulating neutrophils represent only 3% of all neutrophils. A significant depletion of the neutrophil stores would have to occur before there is neutropenia (28). In patients who have decreased marrow stores of neutrophils, there is a report of HSV infection causing neutropenia after bone marrow transplantation (29).

Fas and FasL system represents an important cellular pathway to mediate apoptosis in a large variety of cells and tissues (30,31). Fas is constitutively expressed on human neutrophils, monocytes, activated lymphocytes, and eosinophils (32,33). FasL is expressed predominantly in neutrophils (5) and activated T cells. Our immunofluorescence analysis, showing that Fas and FasL expression increases in neonatal neutrophils infected by HSV-1, indicates that HSV-1-induced apoptosis in neutrophils may also be regulated *via* Fas and FasL pathway. Moreover, this enhanced expression of Fas and FasL in infected cells is not proportional to the number of HSV-1 antigen-expressing cells. This may suggest that even the cells not expressing detectable viral proteins induce a high expression of Fas and FasL proteins. This could be due to the cells being in an infectious environment.

HSV-1-infected cells release sFasL and express more FasL on their membrane surface. sFasL and membrane FasL binds to Fas receptor of noninfected cells, which induce their apo-

ptosis. This could explain how a small proportion of neonatal neutrophils show evidence of infectivity and a large number of cells undergo apoptosis. A similar large effect of HSV infection on T lymphocyte apoptosis has been previously reported (34). In this study, HSV infection resulted in removal by apoptosis of antiviral T lymphocytes *via* Fas receptor activation. This resulted in fratricide of T lymphocytes *via* apoptosis and an increased number of cells affected.

Also, when we treated the infected cells with anti-Fas antibody or with FasL inhibitor, the apoptosis decreased to the level seen in the uninfected, control cells. sFasL in infected neonatal neutrophils was also increased, suggesting that apoptosis of the neutrophils may also be mediated in a paracrine pathway after viral infection.

The clinical relevance of infected neonatal neutrophils having an increase surface expression of Fas and Fas ligand and release of sFasL is supported by previous studies showing that the development of multiple organ failure, specifically hepatic failure was demonstrated in patients with viral infections with increased sFasL levels (35). These findings have also been noted in human and animal studies related to HSV infection (36,37).

Further studies are required to identify the genes responsible of the increase in apoptosis, in particular in the up-regulation of pro-apoptotic and down-regulation of anti-apoptotic proteins. These studies may help better understand the pathogenesis of HSV-1 infection in neonates.

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