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SHORT COMMUNICATION

Apoptosis of cord blood T lymphocytes by herpes simplex virus type 1

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We investigated apoptosis induced by herpes simplex virus type 1 (HSV-1) in cord blood T lymphocytes by using agarose gel electrophoresis, DNA content analysis and the terminal deoxytransferase (TdT)-mediated dUTP nick end-labelling (TUNEL) method. DNA fragmentation and the hypodiploid fraction in the cell cycle were both increased in HSV-1-infected CD4 and CD8 lymphocytes stimulated with phytohaemagglutinin (PHA) compared to mock-infected lymphocytes. The percentage of cells in the S phase was decreased in HSV-1-infected CD4 and CD8 lymphocytes. HSV-1 antigen, glycoprotein D (gD) and regulatory protein ICP27 were detected in 8–18% of the hypodiploid fraction of PHA-stimulated, HSV-1-infected lymphocytes. Apoptosis was induced not only in HSV-1 antigen-expressing cells but also in cells not expressing detectable viral proteins. Addition of anti-Fas antibody, anti-Fas-ligand antibody or a mixture of both had no effect on HSV-1-induced apoptosis, indicating that the Fas–Fas-ligand pathway did not contribute to HSV-1-induced apoptosis.

Herpes simplex virus (HSV) causes severe infection with high mortality in human neonates (Whitley *et al*., 1991). The low frequency of specific T lymphocytes responding to HSV at birth is thought to be responsible for the severity of primary HSV infection in neonates (Hayward *et al*., 1984). Human herpesviruses, such as HSV-1 (Tropea *et al*., 1995), varicella-zoster virus (Sadzot-Delvaux *et al*., 1995) and Epstein–Barr virus (EBV; Kawanishi *et al*., 1993), induce apoptosis. In addition virus-induced apoptosis of lymphoid cells may be a major cause of immunosuppression (Groux *et al*., 1992; Razvi *et al*., 1993). The effect of HSV-1 infection on lymphocytes from neonates has not been fully investigated. In this study, we have investigated the induction of apoptosis by HSV-1 in T lymphocytes from neonates and the relationship between apoptosis and impaired immune function.

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HSV-1 (KOS strain) was grown at an m.o.i. of 0·1 in Vero cells that were cultured in minimum essential medium (MEM, Gibco BRL) supplemented with 10% foetal bovine serum (FBS, Gibco BRL) at 37 °C. After 48 h, the supernatant was collected and cell debris was removed by centrifugation. The supernatant virus stock was stored at −70 °C. The titre of HSV-1 in the virus stock was 2×10^6 p.f.u./ml. Supernatant medium from uninfected Vero cells was used for mock-infected controls.

Cord blood was obtained from the placental end of the cord at full-term birth, and mononuclear cells (MNC) were separated by Ficoll–Hypaque (Histopaque 1077, Sigma) gradient centrifugation. MNC at the interface were collected and washed three times with RPMI-1640 (Gibco BRL) and suspended in RPMI-1640 supplemented with 10% FBS. CD4- and CD8-rich lymphocytes were obtained by using Dynabeads M-450 CD4 and M-450 CD8 (Dynal). These procedures resulted in yields of 95% CD4 and CD8 cells.

Lymphocytes (1×10^6 cells) were centrifuged at 1500 r.p.m. for 5 min. The pelleted cells were resuspended in HSV-1 stock at an m.o.i. of 1·0, or in control supernatant, and then adsorbed for 90 min at 37 °C with occasional mixing. The cells were washed three times with RPMI-1640 to remove unabsorbed virus, and resuspended at a concentration of 1×10^6/ml in RPMI-1640 supplemented with 10% FBS. They were then cultured at 37 °C in 5% CO_2 with or without 5 µg/ml phytohaemagglutinin (PHA, Sigma) for 72 h.

In order to test for DNA fragmentation 1×10^6 mock-infected or HSV-1-infected T lymphocytes were resuspended in 0·5 ml of digestion buffer (100 mM NaCl, 10 mM EDTA, 10 mM Tris–HCl pH 8·0, 0·5% SDS, 200 µg/ml proteinase K) and incubated for 3 h at 50 °C. The DNA was extracted with phenol–chloroform and treated with RNase (100 µg/ml, Sigma). DNA (5 µg) was then mixed with tracking dye (40% sucrose, 0·4% bromophenol blue) and loaded onto 2% agarose gels containing 0·5 µg/ml ethidium bromide. The gels were photographed under UV light.

DNA fragmentation was observed when these cells were cultured with PHA for 72 h but was not observed in mock-infected and HSV-1-infected CD4 and CD8 lymphocytes

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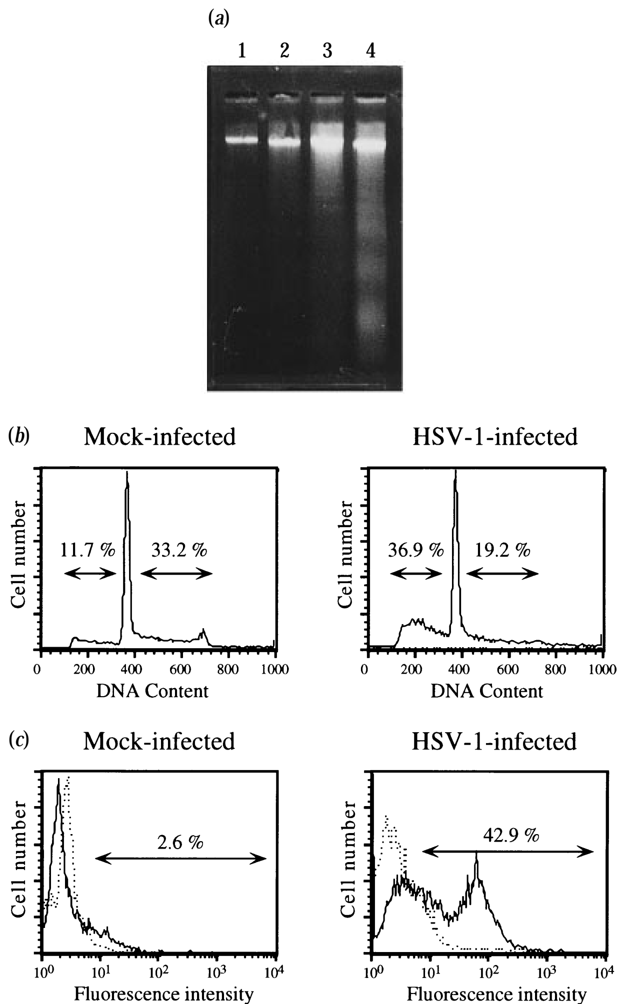

Fig. 1. (*a*) DNA from mock-infected (lanes 1 and 3) and HSV-1-infected CD4 lymphocytes (lanes 2 and 4) was analysed on a 2% agarose gel. Mock-infected and HSV-1-infected CD4 lymphocytes were cultured without stimulation (lanes 1 and 2) and with stimulation by PHA (lanes 3 and 4) for 72 h. (*b*) Cell cycle analysis of mock-infected and HSV-1-infected CD4 lymphocytes. DNA content was analysed by PI staining. Cells were cultured with PHA for 72 h. The percentages of cells present in the hypodiploid fraction and the S phase are indicated to the left and right, respectively. (*c*) Detection of apoptosis by the TUNEL method. Mock-infected and HSV-1-infected CD4 lymphocytes were cultured with PHA for 72 h. Cells were labelled with FITC-conjugated dUTP by TdT (–) or without TdT (···) and analysed by flow cytometry. The percentage apoptosis values are indicated.

cultured without PHA. Typical DNA fragmentation, the 'ladder pattern', was evident in HSV-1-infected CD4 lymphocytes cultured with PHA (Fig. 1*a*, lane 4). Similar results were observed in HSV-1-infected CD8 lymphocytes cultured with PHA for 72 h (data not shown).

Mock-infected and infected lymphocytes were also examined for a hypodiploid DNA content typical of apoptosis (Nicoletti *et al.*, 1991). Cultured lymphocytes (1×10^6 cells) were washed with PBS and resuspended in 75% ice-cold ethanol and kept at −20 °C for 30 min, and then washed with PBS. Cells were incubated with 100 μg/ml RNase at 37 °C for 30 min. The cells were then resuspended in 0·5 ml stain solution [0·1 mg/ml propidium iodide (PI) in PBS; Sigma] at room temperature for 30 min in the dark and analysed with a FACScan (Becton-Dickinson) using the Celfit program (Becton-Dickinson). The percentage of cells in G0/G1, S-G2/M phase or with a hypodiploid DNA content (apoptotic cell nuclei) was calculated using LYSIS software (Becton-Dickinson).

The percentages of cells in the hypodiploid fraction in mock-infected and HSV-1-infected CD4 lymphocytes cultured with PHA were 11·7% and 36·9%, respectively (Fig. 1*b*). Cell cycle distributions of HSV-1-infected and mock-infected cells are summarized in Table 1. The percentages of hypodiploid fraction cells in HSV-1-infected, PHA-stimulated CD4 and CD8 lymphocytes were significantly greater than in the mock-infected controls whereas the percentages of S phase cells in HSV-1-infected CD4 and CD8 lymphocytes cultured with PHA were significantly lower than those of the PHA-stimulated, mock-infected cells. There were no significant differences in the percentages of cells in the hypodiploid fractions of mock- and HSV-1-infected CD4 and CD8 lymphocytes cultured without PHA.

Labelling of DNA strand breaks with fluorescein and analysis by flow cytometry allows for quantitative analysis of apoptosis (Gavrieli *et al.*, 1992). DNA ends (3′OH) generated by DNA fragmentation were nick end-labelled with fluorescein isothiocyanate (FITC)-conjugated dUTP that was introduced by terminal deoxytransferase (TdT) (TUNEL method) with an *in situ* cell death detection kit (Boehringer Mannheim). The cells were fixed in 4% paraformaldehyde in PBS at room temperature for 30 min and washed in PBS. Permeability was enhanced by treatment with 0·1% Triton X-100 in 0·1% sodium citrate for 2 min on ice. Cells were resuspended in the TdT-mediated dUTP nick end-labelling (TUNEL) reaction mixture (FITC-conjugated dUTP) and incubated for 60 min at 37 °C in a humidified atmosphere in the dark. Cells were analysed by flow cytometry using the CELLQuest computer program (Becton-Dickinson).

The percentages of apoptotic cells in mock-infected and HSV-1-infected CD4 lymphocytes cultured with PHA were 2·6% and 42·9%, respectively (Fig. 1*c*) whereas the percentages of apoptotic cells in HSV-1-infected CD4 and CD8 lymphocytes were significantly increased compared with those of mock-infected cells stimulated with PHA (Table 1). There were no significant differences between the numbers of apoptotic cells in mock-infected and HSV-1-infected CD4 and CD8 lymphocytes cultured without PHA.

Expression of HSV-1 antigen, glycoprotein D (gD) and regulatory protein ICP27 was analysed by flow cytometry. Ethanol-fixed cells were stained with 10 μl FITC-conjugated rabbit anti-HSV-1 (DAKO) or FITC-conjugated rabbit immunoglobulin (DAKO) for 1 h at 37 °C. For staining of gD and ICP27, ethanol-fixed cells were incubated with mouse MAb raised against gD [mouse IgG2a; Advanced

Table 1. Apoptosis and cell cycle distribution of HSV-1-infected and mock-infected lymphocytes

HSV-1-infected and mock-infected CD4 and CD8 lymphocytes from cord blood were cultured in the presence or absence of PHA for 72 h. Percentage apoptosis was determined by TUNEL assay and cell cycle was analysed by staining with PI. Data represent mean ± SE from five different experiments.

Cell	Infection	PHA stimulation	% Apoptosis (TUNEL)	Cell cycle distribution			
				Hypodiploid	G0/G1	S	G2/M
CD4	HSV-1	+	40·8 ± 5·6*	38·1 ± 3·2*	42·8 ± 2·6	18·2 ± 1·3*	1·2 ± 0·6
CD4	HSV-1	–	7·2 ± 2·8	18·1 ± 1·2	75·3 ± 1·0	3·9 ± 4·7	2·7 ± 0·5
CD4	Mock	+	6·4 ± 1·6	12·5 ± 3·3	51·4 ± 4·1	36·5 ± 3·8	3·7 ± 1·1
CD4	Mock	–	4·5 ± 2·3	16·2 ± 4·1	78·6 ± 3·6	4·9 ± 1·9	0·3 ± 0·9
CD8	HSV-1	+	54·7 ± 3·8*	58·4 ± 3·3*	31·7 ± 2·2	9·7 ± 2·5*	0·3 ± 2·2
CD8	HSV-1	–	6·8 ± 2·5	13·1 ± 1·5	82·2 ± 1·7	3·4 ± 3·3	1·3 ± 0·7
CD8	Mock	+	8·6 ± 2·6	18·6 ± 1·3	59·7 ± 2·9	20·8 ± 4·1	0·9 ± 0·6
CD8	Mock	–	5·4 ± 3·1	10·6 ± 2·0	85·2 ± 1·5	2·5 ± 2·1	1·7 ± 1·2

* $P < 0.05$ compared with mock-infected cells stimulated with PHA (Wilcoxon signed rank test).

Biotechnologies Incorporated (ABI)], mouse MAb raised against ICP27 (mouse IgG1, ABI) or control mouse IgG1 or IgG2a MAb (DAKO) for 1 h at 37 °C. Cells were washed three times with PBS, incubated with FITC-conjugated affinity-purified $F(ab')_2$ goat anti-mouse IgG (Dako) for 1 h at 37 °C and washed three times with PBS. The cells were then stained with PI and expression of HSV-1 antigen, gD and ICP27 in each cell cycle fraction was analysed by flow cytometry.

The percentages of infected CD4 and CD8 lymphocytes expressing HSV-1 antigen, gD or ICP27 for the whole cell populations and hypodiploid, G0/G1 and S-G2/M fractions are presented in Table 2. Similar results were obtained for the CD4 and CD8 lymphocytes, and expression of the antigens was enhanced by PHA treatment. Only 8–18% of the hypodiploid fractions of PHA-treated cells were positive for antigen expression, in contrast to significantly greater proportions (36–82%) of the corresponding S-G2/M fractions. These data indicate that HSV-1 preferentially replicates in proliferating cells as previously reported (Teute *et al.*, 1983; Braun *et al.*, 1984), and also that apoptosis occurs in cells not expressing detectable HSV-1 antigen.

To determine the effect of anti-Fas and anti-Fas-ligand antibody on apoptosis frequency, anti-human Fas mouse MAb (clone ZB4, mouse IgG1, MBL) and anti-human Fas-ligand MAb (clone4A5, hamster IgG, MBL) were used. Clone ZB4 neutralizes anti-Fas antibody-mediated apoptosis and clone 4A5 neutralizes anti-Fas-ligand-mediated apoptosis (Tanaka *et al.*, 1996). HSV-1-infected CD4 and CD8 lymphocytes were cultured in the presence of anti-Fas antibody (100 ng) or anti-Fas-ligand (100 ng) antibody or a mixture of both for 72 h and the percentage of apoptotic cells was analysed by TUNEL assay. Addition of anti-Fas antibody, anti-Fas-ligand antibody or a mixture of both antibodies did not affect the percentage of apoptotic cells among HSV-1-infected CD4 and CD8 lymphocytes stimulated with PHA (data not shown).

In the present study, we have demonstrated HSV-1-induced apoptosis in CD4 and CD8 lymphocytes from cord blood stimulated with PHA. There is a possibility that soluble factors in the HSV-1 virus stock may induce apoptosis, although this is unlikely because UV- or heat-inactivated virus stock did not induce apoptosis (data not shown). Bovine herpesvirus type 1 (BHV-1) caused cytolysis of activated T lymphocytes and this cytolysis was not associated with virus replication (Griebel *et al.*, 1990). HSV-1 has been shown to induce apoptosis in fresh adult PBMC (Tropea *et al.*, 1995). However, this study did not include investigation of apoptosis in activated T cells or in different T lymphocyte subsets. It has been reported that Marek's disease virus causes apoptosis in CD4 but not CD8 lymphocytes, although the virus can infect both cell types (Morimura *et al.*, 1995). However, the mechanism underlying this difference in effects on CD4 and CD8 lymphocytes is not clear. It is possible that HSV-1 infection may also induce apoptotic cell death in neurogenic cells and that virus latency may result from the blocking of apoptosis, but this does not appear to have been investigated.

HSV inhibits immune cell proliferation in response to mitogenic stimulation (Hayward *et al.*, 1988; Kuo *et al.*, 1993). In our study, cell cycle analysis demonstrated an increase in the hypodiploid fraction and a decrease in S phase cells in HSV-1-infected CD4 and CD8 lymphocytes stimulated with PHA compared with mock-infected lymphocytes. These data indicate that apoptosis induced by HSV-1 infection is one of the mechanisms of suppression of cell proliferation in response to PHA.

Table 2. Expression of HSV-1 antigen, glycoprotein D and regulatory protein ICP27 in cell cycle fraction

HSV-1-infected CD4 and CD8 lymphocytes from cord blood were cultured in the presence or absence of PHA for 48 or 72 h. Cells were incubated with anti-HSV-1, or anti-gD antibody after culture with PHA for 72 h and with anti-ICP27 antibody after culture with PHA for 48 h. Cells were stained with PI following incubation with anti-HSV-1, gD and ICP27 antibody. Data indicate the percentage of cells expressing HSV-1 antigen, gD and ICP27. Representative data are from three different experiments.

Cell	PHA stimulation	Mean from all cell cycles	Expression of HSV-1 antigen		
			Hypodiploid	G0/G1	S-G2/M
CD4	+	17·8	8·2	36·4	82·1
	–	6·0	7·0	5·8	5·0
CD8	+	12·6	11·3	24·0	69·4
	–	5·8	4·0	8·2	6·2
Cell	PHA stimulation	Mean from all cell cycles	Expression of gD		
			Hypodiploid	G0/G1	S-G2/M
CD4	+	31·4	18·7	34·7	87·7
	–	2·1	2·1	1·0	1·0
CD8	+	19·2	7·8	15·1	62·3
	–	2·3	1·8	1·5	20·0
Cell	PHA stimulation	Mean from all cell cycles	Expression of ICP27		
			Hypodiploid	G0/G1	S-G2/M
CD4	+	13·2	12·8	10·2	35·9
	–	1·2	1·1	1·5	1·2
CD8	+	6·3	8·1	3·5	45·2
	–	1·3	1·4	1·1	1·1

The absence of detectable HSV-1 antigens from many of the apoptotic cells amongst the population of infected PHA-stimulated lymphocytes (Table 2) raises the possibility that apoptosis may occur not only in HSV-1-infected cells but also in uninfected cells within the culture. Previous reports have also described apoptosis in both virus-infected and uninfected cells. For example, influenza virus and varicella-zoster virus were demonstrated in apoptotic cells (Hinshaw *et al.*, 1994; Sadzot-Delvaux *et al.*, 1995). On the other hand, cross-linking of the CD4 molecule by the HIV envelope glycoprotein triggers apoptosis in uninfected CD4 lymphocytes (Lu *et al.*, 1994), and lymphocytolysis induced by BHV-1 was not associated with the synthesis of detectable viral proteins (Griebel *et al.*, 1990). We cannot, however, exclude the possibility that apoptosis observed in our studies is induced by a very early event after infection and that viral proteins could not be detected with the methodology used. Detection of viral DNA at the single cell level is needed to clarify whether apoptosis can occur in uninfected cells.

Addition of anti-Fas antibody or anti-Fas-ligand antibody did not affect apoptosis frequency in HSV-1-infected cells, demonstrating that Fas or Fas-ligand do not contribute to HSV-1-induced apoptotic cell death. Therefore, another mechanism of apoptosis induced by HSV-1 must exist. It has been demonstrated that EBV and HSV encode proteins that block apoptosis (Henderson *et al.*, 1991; Chou *et al.*, 1992; Tarodi *et al.*, 1994) and, in general, virus genomes may encode gene products that regulate apoptosis either positively or negatively. Further study will hopefully clarify the mechanism of HSV-1-induced apoptosis of T lymphocytes.

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