Arch Virol (1998) 143: 717–729

Archives of Virology

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Printed in Austria

Fas antigen expression and apoptosis of lymphocytes in macaques infected with simian immunodeficiency virus strain mac

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Accepted November 17, 1997

Summary. To investigate the role of apoptosis in the pathogenesis of HIV infection we used macaques infected with simian immunodeficiency virus (SIV) as a primate model and examined the characteristics of the apoptosis of lymphocytes in SIV mac-infected macaques. In vitro apoptosis was more strongly induced in peripheral blood mononcuclear cells (PBMC) from SIV mac239-infected macaques than those from uninfected controls. We found that the frequency of Fas antigen-positive cells was higher in PBMC from SIV mac-infected macaques than from uninfected controls, and in vitro apoptosis of PBMC was suppressed by an inhibitor of the interleukin-lβ converting enzyme (ICE) family proteases. In biopsied lymph nodes, the number of apoptotic nuclei in T cell-dependent areas was higher in SIVmac-infected macaques than in unifected controls. A higher number of apoptotic nuclei in lymph nodes of SIVmac-infected macaques was observed in the stage of persisent general lymphadenopathy than in those with AIDS-related complex, while there was no significant difference in the extent of apoptosis of cultured PBMC among the SIVmac-infected macaques. These results suggest that in vitro apoptosis is mediated by the Fas/Fas ligand and ICE system and that apoptosis in lymph nodes may be more closely related to the stage of SIVmac infection than is that of cultured PBMC.

Introduction

Chronic infection with human immunodeficiency virus (HIV) in humans leads to progressive depletion of $CD4^+$ T cells, resulting in lethal immune dysfunction. Apoptosis has been proposed as one of the mechanisms of T cell depletion in HIV infection [4]. Spontaneous or activation-induced in vitro apoptosis of peripheral blood mononucler cells (PBMC) and the appearance of apoptotic cells in lymphoid tissues have been observed in HIV type 1 (HIV-1)-infected individuals [4]. Apoptosis of lymphocytes in vivo can be induced under the environments in HIV infection, such as lack of appropriate activation signals to T cells [1, 3], changes in the expression pattern of cytokines [5] and increases in oxidative stress [17]. Furthermore, recent studies have demonstrated that lymphocytes from HIV-1-infected individuals express a high level of Fas antigen and/or Fas ligand, and readily undergo apoptosis in the presence of stimuli for the Fas antigen [7, 12, 15, 19, 20], suggesting that in vitro apoptosis of lymphocytes is mediated by the Fas/Fas ligand system in HIV infection. However, there are many limitations to further investigation of the involvement of apoptosis in the progressive depletion of $CD4^+$ T cells in HIV-infected humans, since it is difficult to obtain clinical samples, especially tissue samples, from the infected individuals throughout the course of HIV infection.

Some animals infected with HIV-equivalent retroviruses develop immunodeficiency diseases, and have been proposed to be non-human models of acquired immunodeficiency syndrome (AIDS). Macaques are commonly used as experimental primates which develop an AIDS-like disease in 1–2 years after infection with simian immunodeficiency virus strain mac (SIVmac) [11].

Spontaneous or activation-induced apoptosis of PBMC has been observed in SIVmac-infected macaques [6, 9], but not in the non-disease-developmental primate models such as African green monkeys [6], mangabeys [21] and chimpanzees [9, 18] infected with SIVagm, SIVsmm and HIV-1, respectively, suggesting that the apoptosis is related to the pathogenicity of SIVmac. In addition, apoptotic cells have been found in the lymph nodes of SIV mac-infected macaques in the late stage of infection[8]. However, the correlation between apoptosis of cultured PBMC and in the lymph nodes of SIVmac-infected macaques, the mechanism of apoptosis induction, and the role of the apoptosis of lymphocytes in the disease progression have not yet been clarified.

In this study, therefore, we studied in vitro apoptosis of cultured PBMC and in vivo apoptosis in biopsied lymph nodes to investigate the correlation between apoptosis and disease progression. We also examined Fas antigen expression on T cells and effects of an inhibitor of the interleukin-lβ converting enzyme (ICE) family proteases on in vitro apoptosis to investigate the involvement of the Fas/Fas ligand system in apoptosis, which have not been previously reported for primate models.

Materials and methods

Subjects

Peripheral blood samples were obtained from three macaques infected intravenously with 1×10^5 $TCID_{50}$ of SIVmac239 (MM 82, MM 83 and MM 105) and three uninfected controls (MM 51, MM 125 and MM 126). Lymph nodes were surgically removed from the three SIVmac-infected macaques at week 60 post infection (p.i.) and from two of the uninfected controls (MM 125 and MM 126). The number of $CD4^+$ cells in PBMC, the cell-associated viral load and the ability to produce antibody against viral envelope glycoprotein (gp130) were monitored longitudinally in the SIVmac-infected macaques before and after infection. Viral load was determined by co-culture of PBMC with $CD4^+$ human T-lymphoid cell line M8 166 cells, and antibody production was evaluated by Western blotting analysis, as described elsewhere [10].

Cell culture

PBMC were isolated from heparinzed blood by centrifugation on a Ficoll-Hypaque (Pharmacia. Uppsala, Sweden) density gradient, resuspended in RPMI 1640 (Nikken Biomedical Lab., Kyoto, Japan) supplemented with 10% heat-inactivated fetal calf serum (FCS; Cam Sera, Ontario, Canada) and 60 μg/ml kanamycin, and 5×10^5 cells/well were cultured in 24-well plates. In some experiments, $CD4^+$ or $CD8^+$ cells were depleted from PBMC using immunomagnetic beads coated with anti-mouse lgG (DYNAL A.S, Oslo, Norway) following treatment of the cells with mouse monoclonal antibody (MAb) against CD4 or CD8 (Nichirei, Tokyo, Japan) before cultivation. Ninety six percent of $CD4^+$ cells and 85% of $CD8^+$ cells on the average were depleted as assessed by flow cytometry (FACScan, Becton Dickinson, San Jose, CA).

Detection of apoptosis of PBMC

Apoptotic cells exhibiting characteristic nuclear chromatin and DNA fragmentation were detected by three methods. 1. Under a fluoresecence microscope, nuclear changes in PBMC were observed after incubation of 10^6 cells with a nuclear dye, Hoechst 33342 (20 μg/ml), for 15 min at 37 °C. 2. Apoptotic cells were quantified by flow cytometric analysis of PBMC stained with a nuclear dye, propidium iodide (PI). Two million cells were fixed in 50 μl of acetone/methanol (1:1) for 10 min at −20 °C, washed once with 30% cold methanol and once with phosphate-buffered saline (PBS), and resupended in 50 μg/ml RNase solution. After a 30-min incubation at 37 °C the cells were resuspended in 50 μl of 20 μg/ml PI solution and incubated at 4 °C for 90 min. In analysis of stained cells by flow cytometry, apoptotic cells were represented by a characteristic distinct peak with reduced fluorescence intensity. 3. Quantitative analysis of DNA fragmentation was performed by agarose gel electrophoresis of DNA extracted from PBMC. Two million cells were incubated with 60 μl of lysis buffer (10 mM Tris-HCI pH 7.4, 10 mM EDTA pH 8.0 and 0.5% Triton X-100) for 10 min at 4 °C. The cell lysate was centrifuged for 25 min at 1.5×10^3 **g** to remove the debris and then RNase (0.5 mg/ml) was added to the supernatant. After a l-h incubation at 37 °C, proteinase K (0.5 mg/ml) was added and the lysate was digested for a further 1 h. The extracted DNA was electrophoresed on a 2% agarose gel in TBE buffer (45 mM Tris- borate and 1 mM EDTA pH 8.0), stained with 0.5 μg/ml ethidium bromide and visualized under UV light.

Inhibition of ICE family proteases

A peptide inhibitor of ICE-family proteases, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk; Kamiya Biochemical Co., Thousand Oaks, CA), was dissolved

in DMSO (100 mg/ml) and stored at −80 °C. Freshly isolated PBMC from SIVmac-infected and uninfected macaques were cultured, as described above, for 24 h in the absence or presence of the ICE inhibitor (0.1 mg/ml).

Analysis of Fas antigen expression

Fas antigen expression on $CD4^+$ and $CD8^+$ cells in PBMC was determined by flow cytome tric analysis. Monoclonal anti-Fas antibody was prepared as previously described [22] and confirmed to recognize Fas antigen of macaques. Freshly isolated PBMC were preincubated with 5% autologous serum to avoid nonspecific staining and incubated with 20 μg/ml of mouse anti-Fas MAb, and then, with 10 μg/ml of fluorescein-isothiocyanate (FITC)-conjugated anti-mouse lgG (Nichirei). Next, cells were incubated with 5% normal mouse serum to block unoccupied sites of the anti-mouse lgG, and finally stained with a mouse MAb, phycoerythrin (PE)-conjugated anti-CD4 (diluted 1:25, Nichirei) or FITC-conjugated anti-CD8 (diluted 1:25, Becton Dickinson and Co.). Each incubation step was performed for 30 min on ice. After fixation in 10% formaldehyde to inactivate the virus, cells were analyzed by flow cytometry.

Histochemical analysis of lymph nodes

Lymph nodes removed surgically were immediately fixed in 4% paraformaldehyde in PBS and embedded in paraffin. The lymph nodes were then sectioned to 6-μm thickness and placed onto slide glasses coated with 3-aminopropyl-triethoxysilane.

An in situ apoptosis detection kit using peroxidase, Apop Tag plus (ONCOR, Gaithersburg, MD), was used to detect free 3′-OH ends of cleaved DNA in the lymph node sections. The apoptotic nuclei were quantified by counting TdT-positive nuclei in the paracortex and germinal centers under a microscope. The total area analyzed was dependent on the size of each compartment; 2.5×10^6 μm^2 in the paracortex of lymph nodes of SIVmac-infected and uninfected macaques, and 7.5×10^5 μm^2 and 2.5×10^5 μm^2 in the germinal centers of lymph nodes of SIVmac-infected and uninfected ones, respectively.

Statistical significance was assessed by the unpaired t-test.

Results

Clinical state of SIVmac-infected macaques

Immunological and virological states of three SIVmac-infected macaques (MM82, MM83 and MM105) were monitored before and after infection by monitoring number of $CD4^+$ cells in PBMC, the ability to produce antibody against the virus envelope protein gp 130 (Fig. 1) and viral load. During the period between weeks 47 and 71 p.i., MM82 and MM105 showed persistent general lymphadenopathy (PGL) and decrease in the number of $CD4^+$ cells in PBMC (337 and 550 cells/μl at week 47, and 118 and 290 cells/μl at week 71 p.i. in MM82 and MM105, respectively), indicating that these two macaques had progressed to the stage of AIDS-related complex (ARC). On the other hand, MM83 showed PGL only and the number of $CD4^+$ cells in PBMC was within the normal range (869 and 1850 cells/μl during weeks 47 and 71 and 71 p.i.) Moreover, antibody against SIVmac gp130 had disappeared in MM82 and MM105, but was present in serum of MM83 during this period. Apoptotic analysis study was performed within this period (shadowed in Fig. 1).

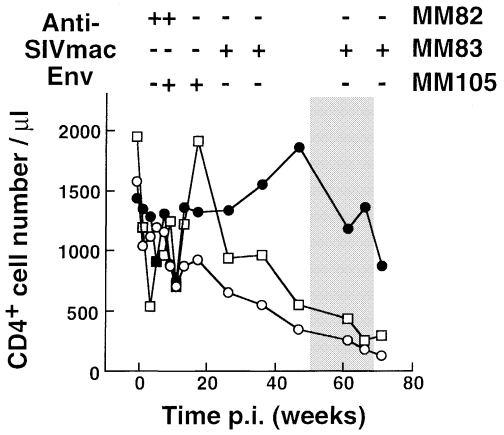


Fig. 1. Change in peripheral $CD4^+$ cell number and ability to produce antibody against SIVmac Env after SIVmac infection. ○ MM82; ● MM83; □ MM105. Shadow represents the period of apoptotic analysis

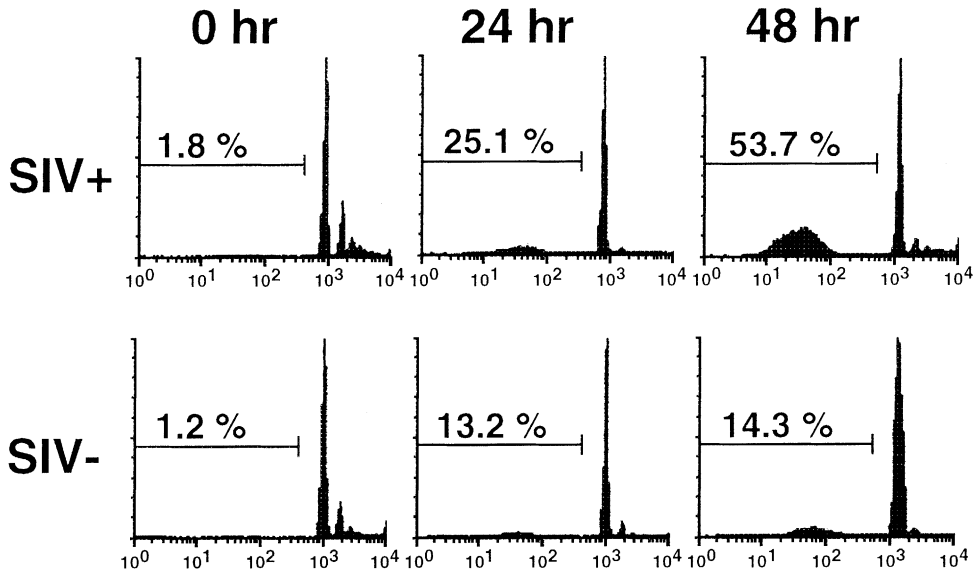


Fig. 2. Quantification of in vitro apoptosis of PBMC from macaques. Representative flow cytometric analysis for SIVmac-infected (MM83, top) and uninfected macaques (MM126, bottom) is shown. Gates are set on apoptotic populations

Virus particles were fairly detected in all the macaques at every test point after infection (data not shown).

In vitro apoptosis of lymphocytes from SIVmac-infected macaques

Apoptotic cells in PBMC were quantified by flow cytometric analysis with PI staining (Fig. 2). The percentage of apoptotic cells was significantly higher in cultured PBMC from SIVmac-infected macaques (25.1–31.6% at 24 h and 46.9–56.8% at 48 h) than in those from uninfected controls (6.1–14.6% at 24 h and 12.2–15.9% at 48 h) ($p=0.008$ and $P=0.001$, respectively) (Table 1). In the

Table 1. In vitro apoptosis of PBMC from SIVmac-infected and uninfected macaques

No.	SIV	% of apoptotic cells		
		0 h	24 h	48 h[a]
MM82	+	6.3	31.6[b]	56.8[c]
MM83	+	1.8	25.1[b]	53.7[c]
MM105	+	5.5	26.3[b]	46.9[c]
MM51	−	ND	6.1[b]	12.2[c]
MM125	−	0.4	14.6[b]	15.9[c]
MM126	−	1.2	13.2[b]	14.3[c]

[a]Cultivation time,
[b]p=0.008,
[c]p=0.001,
ND Not determined

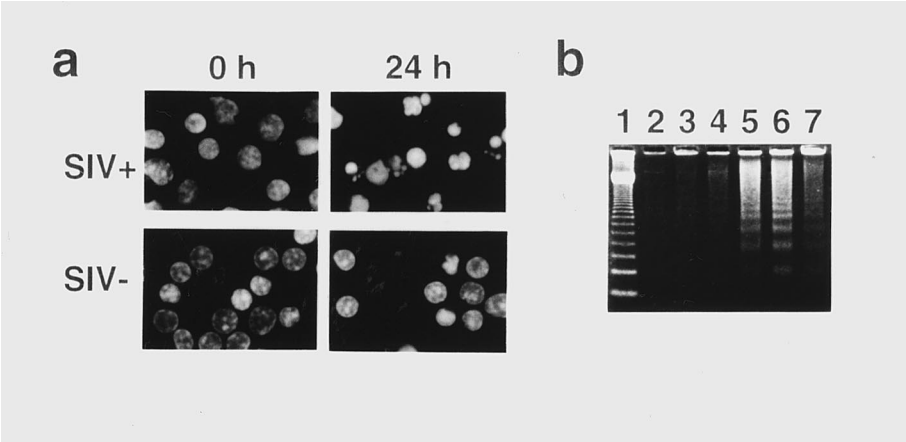


Fig. 3. Apoptotic nuclei and fragmented DNA. **a** Representative views seen upon fluorescence microscopic analysis of fresh (left) or 24 h cultured PBMC (right) from SIVmac-infected (MM82, top) or uninfected macaques (MM125, bottom). **b** Agarose gel electrophoresis of DNA extracted from PBMC from SIVmac-infected macaques (*5–7*, MM82, 83 and 105, respectively) and from uninfected ones (*2–4*, MM51, 125 and 126, respectively) after 8 h culture. *1* contains DNA markers (123bp DNA ladder)

SIVmac-infected macaques, there was no significant difference in the percentage of apoptotic cells between MM105 and MM82 in the stage of ARC and MM83 in the stage of PGL (Table 1).

Under a fluorescence microscope, nuclear condensation was more often observed in cultured PBMC from SIVmac-infected macaques than those from uninfected controls after staining with Hoechst 33342 (Fig. 3a). The percentage of the apoptotic cells in the cultured PBMC was 20–30% in SIVmac-infected macaques. and less than 10% in uninfected controls.

Table 2. In vitro apoptosis of $CD4^+$-depleted and $CD8^+$-depleted PBMC from SIVmac-infected macaques after 24 h cultivation

No.	% of apoptotic cells	
	CD4-	CD8-
MM82	27.0	36.7
MM83	33.9	43.5
MM105	30.9	29.8

Table 3. Fas antigen expression on $CD4^+$ and $CD8^+$ cells from SIVmac-infected and uninfected macaques

No.	SIV	$CD4^+$	$CD8^+$
MM82	+	87.7[a]	98.0[b]
MM83	+	75.4[a]	98.5[b]
MM105	+	75.0[a]	98.4[b]
MM51	−	57.2[a]	78.3[b]
MM125	−	53.6[a]	85.7[b]
MM126	−	48.3[a]	73.4[b]

[a] $p=0.010$
[b] $p=0.033$

DNA extracted from cultured PBMC was analyzed on an agarose gel. After 8-h cultivation, fragmented DNA was detected in PBMC from SIVmac-infected macaques, but not in PBMC from uninfected controls (Fig. 3b).

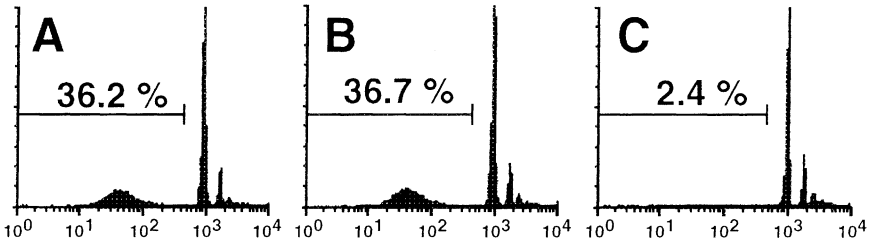
To examine the subset of apoptotic T cells in SIVmac-infected macaques, $CD4^+$-rich and $CD8^+$-rich cells were prepared from PBMC by depletion of $CD8^+$ and $CD4^+$ cells, respectively, using immunomagnetic beads and cultured for 24 h as described above. Apoptosis was detected both in $CD4^+$-rich (29.8–43.5%) and $CD8^+$-rich (27.0–33.9%) cell fractions (Table 2).

Contribution of the Fas antigen and ICE-family proteases to apoptosis

The percentage of Fas antigen-positive cells was significantly higher both in $CD4^+$ and $CD8^+$ cells from SIVmac-infected macaques (75.0–87.7% in $CD4^+$ cells and almost 100% in $CD8^+$ cells) than in those from uninfected controls (48.3–57.2% in $CD4^+$ cells and 73.4–85.7% in $CD8^+$ cells) ($p=0.01$ and $p=0.033$, respectively) (Table 3).

An ICE inhibitor, Z-VAD-fmk, reduced the ratio of in vitro apoptosis of PBMC of two SIVmac-infected macaques from 36.2–40.5% to 2.4–3.4% and one

a



b

No.	SIV	Medium	DMSO	Inhibitor
MM82	+	40.5	41.9	3.4
MM83	+	36.2	36.7	2.4
MM126	−	10.8	11.0	0.7

Fig. 4. Suppression of in vitro apoptosis, by an ICE inhibitor, Z-VAD-fmk, of PBMC from macaques. **a** A representative flow cytometric analysis in SIVmac-infected macaques (MM83). BNMCs were cultured in growth medium only (*A*), containing 0.1% DMSO (*B*), or 0.l mg/ml ICE inhibitor (*C*) **b** Summary of apoptosis inhibition by Z-VAD-fmk. Values in **a** and **b** are percentages of apoptotic cells

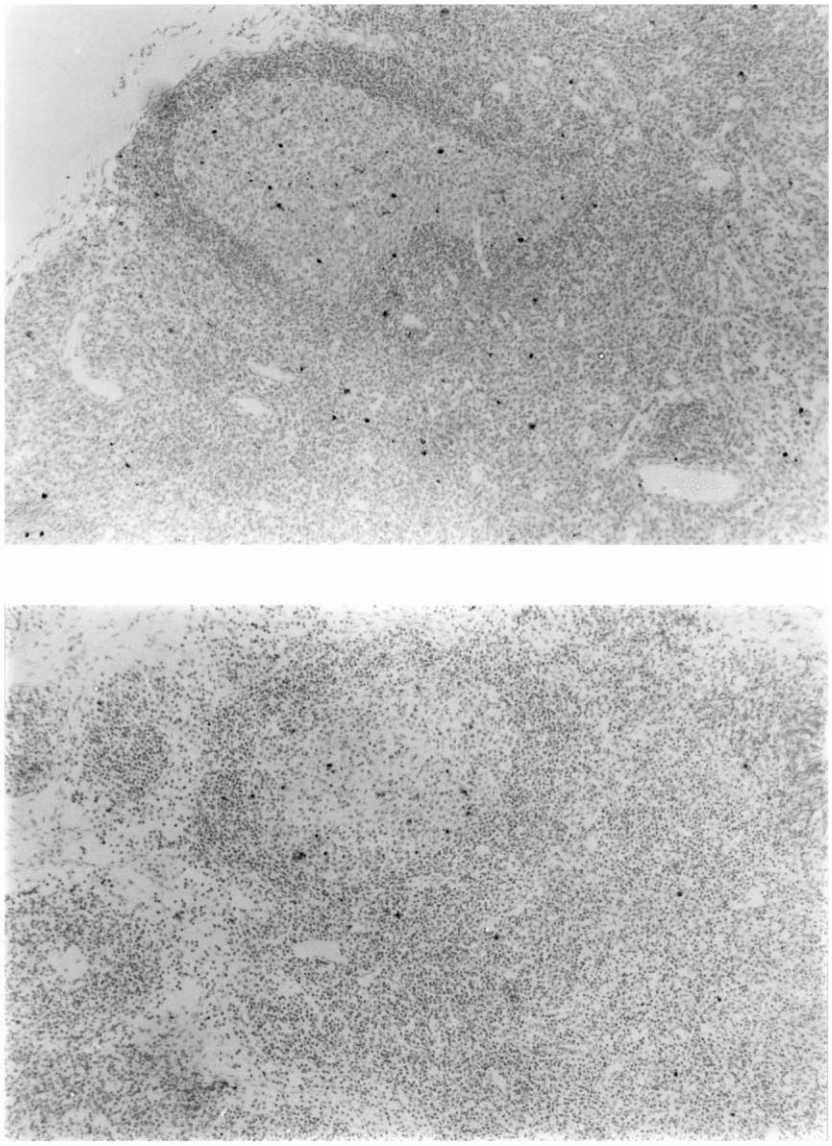
uninfected macaque from 10.8% to 0.7% (Fig. 4). The percentage of apoptotic cells was not changed by the solvent of Z-VAD-fmk, DMSO, alone (Fig. 4).

Histochemical analysis of lymph nodes

In haematoxylin-eosin staining of the lymph node sections from the SIV mac-infected macaques, follicular hyperplasia and involution, which are characteristic changes in the morphology of lymph nodes in HIV or SIVmac infection were observed (data not shown). Then, apoptotic nuclei were detected by TdT assay in lymph node sections from the SIVmac-infected and uninfected macaques. TdT-positive nuclei were observed both in the paracortex and germinal centers in the lymph node sections from the SIVmac-infected macaques (Fig. 5 a A), whereas they were mostly confined to the germinal centers in the section from the uninfected controls (Fig. 5 a B). The number of apoptotic nuclei in the paracortex, a T-cell zone, was from 3- to 6-fold higher in SIVmac-infected lymph nodes

Fig. 5. TdT analysis of lymph nodes from macaques. **a** Representative view of lymph node sections from SIVmac-infected (MM83, *A*) and uninfected macaques (MM125, *B*). TdT-positive nuclei are observed as black spots. Morphology of lymph nodes was assessed by counter staining of the sections with methyl green. Second follicles are seen at the left-top side of *A* and *B*. **b** Summary of the number of apoptotic nuclei per area in germinal centers (*GC*) and in the paracortex (*PC*)

a



b

No.	SIV	GC	PV
MM82	+	33.9[a]	11.8
MM83	+	46.3	15.5
MM105	+	33.2	8.3
MM125	−	32.8	2.8
MM126	−	34.8	2.8

GC Germinal center, *PC* Paracortex
[a]Number of apoptotic nuclei/1 × 10^5 μm^2

(8.3–15.5/area) than in uninfected ones (2.8/area), whereas in germinal centers, there was no significant difference in the number of apoptotic nuclei between the two groups (33.2–46.3 vs 32.8–34.8/area) (Fig. 5b). Of the three SIVmac-infected macaques, MM83 with a normal $CD4^+$ cell count in peripheral blood exhibited a higher number of apoptotic nuclei both in the paracortex and germinal centers than did MM82 and MM105, whose $CD4^+$ cell counts in peripheral blood were decreased, though statistical significance could not be assessed due to the insufficient number of the SIVmac-infected macaques in different stages of the disease.

Discussion

Three macaques infected with a pathogenic strain of SIV, SIVmac239, were used in this study. During the analysis of apoptosis, two of them (MM82 and MM105) were estimated to be in the stage of ARC as a result of exhibiting PGL and reduced $CD4^+$ cell counts in peripheral blood, and their ability to produce antibody against SIVmac Env gp130, which is one of the indicators of disease progression [13], was lost, indicating that they would develop the full-blown disease in the near future. After the series of analyses, MM 105 and MM82 died from opportunistic infections such as *Pneumocystis carinii* and acute respiratory distress syndrome at 75 and 85 w.p.i., respectively. On the other hand, the other one (MM83) was in the stage of PGL and still exhibited the ability to produce the antibody.

Spontaneous in virto apoptosis was induced more strongly in PBMC of the SIVmac-infected macaques than in those of uninfected ones, previously reported [6, 9]. However, there was no significant difference in the extent of in vitro apoptosis between MM105 and MM82 in the stage of ARC and MM83 in the stage of PGL. No significant relationship between the extent of in vitro apoptosis and the stage of the disease has been previously reported either [6, 9]. Thus, PBMC are suggested to be continuously activated and primed for apoptosis in the SIVmac-infected macaques despite of the stage of infection.

Since the number of $CD4^+$ cells was more extensively decreased than that of $CD8^+$ cells in peripheral blood in the SIVmac-infected macaques (MM82 and MM105, data not shown), we clarified whether in vitro apoptosis of PBMC occurred more extensively for $CD4^+$ cells than $CD8^+$ cells. In vitro apoptosis of both $CD4^+$ and $CD8^+$ cells occurred, but there was no significant difference in the ratio of apoptosis between the two subsets. A similar discrepancy has also been observed in other primates and humans [6, 14]. Thus, $CD4^+$ and $CD8^+$ cells are suggested to be equally primed for apoptosis in the SIVmac-infected macaques. However, being different from in vitro culture, selective induction of apoptosis and/ or a failure in reproduction of $CD4^+$ cells may occur in vivo such as by the cross-linking of CD4 molecules by viral envelope protein gp130 and anti-gp130, as suggested in HIV-infected humans [2, 4, 23].

To clarify the mechanism of in vitro apoptosis of lymphocyes, Fas antigen expression and the involvement of ICE family proteases in apoptosis were investigated in macaques for the first time. The frequency of Fas antigen-positive cells was significantly higher in $CD4^+$ and $CD8^+$ cells from SIVmac-infected

macaques than in those from uninfected controls, in agreement with the results of previous studies on HIV-1 infected humans [17, 12, 19]. An ICE protease inhibitor, Z-VAD-fmk, almost completely suppressed the in vitro apoptosis of PBMC, indicating that the apoptosis was mediated by the ICE family proteases. These are the first observations which suggest the involvement of the Fas/Fas ligand and ICE system in the spontaneous in vitro apoptosis of PBMC in SIVmac infection. Thus, the in vitro apoptosis of PBMC of SIVmac-infected macaques may be enhanced by the overexpression of Fas antigen and subsequent activation of the ICE family proteases.

The in situ TdT assay revealed more extensive distribution of apoptotic nuclei in the paracortex, a T cell dependent area, of lymph nodes from SIVmac-infected macaques than in that of lymph nodes from uninfected controls. Similar findings were obtained in lymph nodes of HIV-1-infected humans [8, 16]. It is noted that paracortical cells were relatively sparse in MM82 and MM105 compared with in MM83 in HE staining of the lymph node sections (data not shown), and that among the three SIVmac-infected macaques, MM83 with a normal $CD4^+$ cell count in peripheral blood exhibited a higher number of apoptotic nuclei both in the paracortex and germinal centers than did MM82 and MM105, whose $CD4^+$ cell counts in peripheral blood were decreased. These results suggest that induction of the apoptosis of T-cells in lymph nodes may cause a subsequent decrease in the number of $CD4^+$ cells in peripheral blood, though the difference in the number of apoptotic cells in the lymph nodes in the SIVmac-infected macaques in not statistically significant and may only reflect the decrease in the total number of paracortical cells. It is therefore necessary to monitor the apoptosis in lymph nodes longitudinally using this SIVmac-infected macaque model to clarify the correlation between apoptosis and the disease progression.

Thus, our findings in this study suggest that in vitro apoptosis is mediated by the Fas/Fas ligand and ICE system and that apoptosis in lymph nodes may be more closely related to the stage of SIVmac infection than is that of cultured PBMC.

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Received October 2, 1997