T-Cell Death by Apoptosis in Vertically Human Immunodeficiency Virus-
Infected Children Coincides With Expansion of CD8+/Interleukin-2
Receptor-/HLA-DR+ T Cells: Sign of a Possible Role for
Herpes Viruses as Cofactors?

By Roger P. Lauener, Silke Hüttner, Marlyse Ruisson, Johann P. Hossle, Manuela Albisetti, Jean-Marie Seigneurin, Reinhard A. Seger, and David Nadal

One mechanism proposed to play a role in T-cell depletion in human immunodeficiency virus (HIV) infection is apoptosis (activation-induced cell death). We assessed whether apoptosis is related to activation of T cells in vivo and its possible triggers. DNA was extracted from peripheral blood mononuclear cells (PBMC) taken from 16 vertically HIV-infected children and 9 HIV-negative children born to HIV-positive mothers (controls) and tested by agarose gel electrophoresis for the presence of DNA fragments specific for apoptosis. Signs of apoptosis were found on in vitro culture of PBMC from 12 of 16 HIV-infected children, but not in PBMC from the nine controls. Eleven of the 12 HIV-infected children, with apoptosis showed an elevated (>15%) proportion of CD3+/CD8+/HLA-DR+ cells. This was due to an increased proportion of CD8+/HLA-DR+ cells, as shown in 7 of 7 further tested patients. In none of the probands an increased (>5%) proportion of IL-2 receptor expressing CD3+ cells was found. T cells undergoing apoptosis were preferentially of the CD8+ phenotype. Expansion of circulating CD8+/Interleukin-2 receptor (IL-2R)/HLA-DR+ T cells is known to occur during active infection with herpes viruses. To investigate the possible role of herpes viral coinfections for apoptosis in HIV infection, we focused on Epstein-Barr virus (EBV) as an example for a herpes virus usually acquired during childhood. In 10 of 12 patients with apoptosis, we found increased levels of EBV genome in PBMC and/or tissues, indicating active EBV replication. By contrast, no increased burden of EBV was found in the four HIV-infected patients without apoptosis or in the controls. Our data indicate that in children the occurrence of apoptosis in HIV infection is closely related to activation of CD8+ T cells. Furthermore, primary infection with or reactivation of herpes viruses, such as EBV, may substantially contribute to such T-cell activation and the ensuing apoptosis. Additional studies are warranted to evaluate the contribution of herpes virus-triggered apoptosis to the T-cell loss leading to the acquired immunodeficiency syndrome.

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MATERIALS AND METHODS

Patients. Blood samples were taken on the occasion of routine controls from children born to HIV-positive mothers followed up in our clinic. Children were included in the study only if they were over 1 year of age and the status of HIV infection was documented by at least two cultures for HIV and by at least two polymerase chain reaction (PCR) assays for the detection of HIV genome. Children born to HIV-infected mothers and documented to be HIV-negative as described above served as controls. Additionally, blood was drawn from pediatric patients suffering from acute EBV infection (familial mononucleosis) as diagnosed by the clinical symptoms and the detection of IgM specific to EBV capsid antigen.

Isolation and culture of PBMC. PBMC were isolated from heparinized venous blood of patients and controls by Ficoll-Hypaque density gradient centrifugation. The cells were then resuspended in RPMI 1640 medium (GIBCO BRL, Basel, Switzerland) supplemented with 10% AB+ serum (ICN/Flow Laboratories, Allschwil, Switzerland), 2 mmol/L glutamine, 50 μg/mL streptomycin, and 100 U/mL penicillin (Sigma, Buchs, Switzerland) and cultured at a concentration of 10^6 cells/mL for 16 hours to 24 hours at 37°C in a 5% CO2 atmosphere.

Flow cytometry analysis. Freshly isolated PBMC (0.5 × 10^6 cells/mL) were stained using phycoerythrin (PE) or fluorescein isothiocyanate (FITC) conjugated anti-CD3 (anti-Leu-4), anti-CD4...
and anti-HLA-DR (L243) (Becton Dickinson (anti-Leu-3a), anti-CD8 (anti-Leu-2a), anti-CD25, and anti-HLA-DR (L243) (Becton Dickinson & Co, Basel, Switzerland) monoclonal antibodies (MoAbs) following the instructions of the manufacturer. Murine IgG MoAbs of unrelated specificity conjugated to PE and FITC (Becton Dickinson was used as controls. In some cases, whole blood samples were stained after lysis of red cells following the manufacturer’s instructions. Percentages of positive cells and mean fluorescence intensity were analyzed by a FACScan (Becton Dickinson & Co) gating on the lymphocyte population, as defined by forward and side light scatter.

Analysis of the phenotype of apoptotic cells was performed using flow cytometry as described, taking advantage of the fact that apoptotic and nonapoptotic cells form distinct cell populations in light scattergrams, reflecting their smaller size and nuclear fragmentation.

PBMC were isolated and cultured for 16 to 24 hours as described above before staining with MoAbs. Expression of cell surface markers was then analyzed by flow cytometry. Gates were set on the cell populations consisting of smaller, apoptotic cells (region 1), and on larger, nonapoptotic cells (region 2). Expression of CD45 was found to be decreased in region 1, confirming the presence of apoptosis in the cells gated in this region.4 Expression of cell surface markers was compared between the cells in region 1 and region 2. No distinct population of smaller cells corresponding to region 1 was found in three experiments assessing freshly isolated cells from healthy controls or HIV-positive patients, or in cultured (16 to 24 hours) PBMC from healthy controls or HIV-positive, apoptosis-negative patients.

DNA extraction and agarose gel electrophoresis. DNA extraction from PBMC was performed following the protocol 1 in Sambrook et al. In brief, 5 × 10^6 to 15 × 10^6 PBMC were resuspended in 750 μL of ice cold extraction buffer (10 mmol/L Tris/HCl pH 8.0; 0.1 mmol/L EDTA pH 8.0; 20 mg/mL RNase A; 0.5% sodium dodecyl sulfate (SDS)), and then incubated for 1 hour at 37°C. A total of 10 μL of a proteinase K stock solution (10 mg/mL) was added before the suspension of lysed cells was placed into a water bath for 3 hours to 12 hours at 50°C. Samples were subsequently extracted three times with an equal volume of saturated phenol pH 7.9; 0.2 mL 10 mmol/L ammonium acetate was added, and DNA was precipitated with 2 mL ethanol for 2 hours at 12 hours at −20°C. DNA was pelleted by centrifugation at maximal speed for 30 minutes in the microfuge in the cold, washed with 70% ethanol, and briefly dried in a vacuum centrifuge. Finally, DNA pellets were dissolved in 0.2 mL TE (10 mmol/L Tris, 1 mmol/L EDTA) pH 8.0. The DNA content was calculated from absorbance of the DNA at 260 nm and equal amounts were loaded onto a 1% agarose gel. After electrophoresis, DNA was visualized by staining with ethidium bromide. Appearance of the ladder pattern characteristic for apoptosis on loading 10 μg of DNA was interpreted to indicate that apoptosis had occurred in a pathologically elevated percentage of cells.

Detection of EBV in PBMC. For semiquantitative PCR, PBMC were homogenized in NET buffer (10 mmol/L Tris-HCl, pH 8; 10 mmol/L EDTA, pH 7.4; 100 mmol/L NaCl), and digested with proteinase K (100 μg/mL) in the presence of 0.5% SDS at 37°C. DNA was obtained after phenol/chloroform extraction and precipitated with ethanol (2.5 vol) in the presence of sodium acetate (3 mol/L, 0.1 vol). After centrifugation at 7,000g for 30 minutes, DNA was dissolved in sterile water and stored at −20°C.

The Namalwa cell line, which contains two copies of EBV-DNA sequences per cell was used for the control range. The first range point contained 1 μg of Namalwa (1 μg = 150,000 cells = 300,000 copies). Serial 10-fold dilutions were performed to obtain a range of 1 μg to 1 pg of EBV-DNA sequence (300,000 copies to 0.3 copies). The range points (0.1 μg to 1 pg) were mixed with 1 μg DNA of BJAB (an EBV-negative Burkitt’s lymphoma cell line). For DNA amplification, we used a pair of primers (BC1 [nucleotides position 4010-4029] and BC2 [nucleotides position 4111-4130] from Dr M. Perricaudet, Institute Gustave Roussy, Villejuif, France)

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Fig 1. Apoptosis in PBMC from children infected with HIV. Agarose gel electrophoresis of genomic DNA extracted from PBMC of a representative healthy HIV-negative child (Contr, no. 6 from Table 1) and from two representative patients with HIV infection (proband no. 10 and 2 from Table 1). DNA was extracted from freshly isolated PBMC (culture −) or after 16 to 24 hours of culture (culture +). A total of 10 μg of DNA was loaded on each lane. Note the typical ladder pattern of apoptosis of cultured PBMC from patient no. 24. Mw, molecular weight markers.
within the BamHI C sequence of the B95-8 EBV cell line to amplify a fragment of 120 bp. The Namalwa range points and 1 pg of patient’s DNA were used for PCR. DNA amplification was performed in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) for one cycle consisting of denaturation (94°C; 15 minutes), 40 cycles consisting of denaturation (94°C; 90 seconds), annealing (50°C; 90 seconds), and primer extension (72°C; 120 seconds). After the last cycle, primer extension was continued for 8 minutes to allow all templates to be completed. After electrophoresis on a 2% agarose gel, DNA was transferred onto positively charged nylon membrane (Hybond, Amersham, Arlington Heights, IL) and hybridized with a 32P-ATP-labeled probe (BC3 [nucleotides position 4051-4090] from Dr M. Perricaudet). With this semiquantitative PCR, 10 pg of Namalwa DNA (= 3 copies) can be detected.

Detection of EBV in tissues. For the detection of EBV by PCR in lymphoma, tonsils, or oral hairy leukoplakia cells, we used a pair of primers within the long internal repeat sequence to amplify a fragment of 335 bp. The PCR products were blotted onto nitrocellulose membranes. The immobilized PCR products were specifically detected by hybridization using a digoxigenin-labeled internal sequence of 253 bp as probe, followed by incubation with alkaline phosphatase-conjugated antibody to digoxigenin and color precipitation of nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP).

Antibodies to EBV. The IgG serologic responses against EBV capsid antigen (VCA), EBV early antigen (EA), and EBV nuclear antigen (EBNA) and the IgM response to VCA were determined with standard indirect immunofluorescence techniques. A positive IgG VCA antibody concentration was defined as a titer > 1:25; an elevated EA antibody response was defined as a titer > 1:10; an elevated IgG VCA antibody concentration was defined as a titer > 1:50; and an elevated EA antibody response was defined as a titer > 1:40. A positive EBNA antibody response was defined as a titer > 1:5.

Statistical analysis. Numbers were expressed as mean ± SE. Statistical significance was evaluated by Student’s t-test for unmatched pairs.

**RESULTS**

Apoptosis occurs in PBMCs of vertically HIV-infected children when cultured in vitro. Analysis of DNA ex-
Fig 2. Apoptosis and T-cell activation. The individual data points represent the percentage of CD3+/HLA-DR+ lymphocytes of nine healthy HIV-negative children (controls), four HIV-infected children showing no signs of apoptosis (HIV infection, apoptosis –), 12 HIV-infected children with signs of apoptosis (HIV infection, apoptosis +), and three patients with acute infectious mononucleosis (IM). The hatched areas indicate the mean values ± standard error.

Extracted from PBMCs from nine healthy controls showed no signs of apoptosis after culturing the cells for 16 to 24 hours (Fig 1 and Table 1). However, in 12 of 16 HIV-infected children, 16 to 24 hours culture of the PBMCs in the absence of any exogenously added stimulus was ensued by the “lad-der” pattern in DNA gel electrophoresis characteristic for apoptosis. Experiments with both controls and HIV-infected children showed no signs of apoptosis in freshly isolated PBMC not subjected to prior culturing, confirming earlier reports.11-13

To define the factors associated with apoptosis, we first compared the results of our investigations for apoptosis with laboratory data from our patients. Independent of their apoptosis-status, HIV-infected children had a statistically significant lower ratio of CD4+/CD8+ T cells than the controls: 2.0 ± 0.3 (mean ± SE) for the controls versus 0.5 ± 0.1 (0.4 ± 0.2) for the patients with (without) signs of apoptosis (P < .01 for both apoptosis-positive and apoptosis-negative children when compared with controls). The mean age of our controls (3.1 ± 0.7 years) was lower than that of apoptosis-positive (6.2 ± 1.1 years) and apoptosis-negative (5.5 ± 1.3 years) HIV-infected patients. However, none of these parameters differed significantly between those HIV-infected children displaying signs of apoptosis and those who did not.

Apoptosis occurs in lymphocytes activated in vivo. Apoptosis of mature lymphocytes has been observed to occur after cellular activation14-16; however, we have found apoptosis in PBMC not exposed to in vitro stimuli, e.g., mitogens. This finding is suggestive for activation already within the host. To explore this possibility, we examined the patients' PBMCs ex vivo for the expression of activation-associated markers by flow cytometry. The receptor for interleukin-2 (IL-2R; CD25) is expressed on T cells after activation.

Fig 3. Activated T cells are mainly of the CD8+ phenotype. Freshly isolated PMBC were analyzed by flow cytometry for the expression of HLA-DR as a marker for activation on CD3+, CD4+ and CD8+ T cells. PBMC from a healthy, HIV-negative child (a; proband no. 2, Table 1), an HIV-positive, apoptosis-negative child (b; proband no. 11, Table 1), and an HIV-positive, apoptosis-positive child (c; proband no. 18, Table 1) are shown as representative examples. Data are listed in Table 1.
However, no patient or any control displayed >5% CD3+/IL-2R+ lymphocytes on examination of PBMC ex vivo (data not shown); this was not due to a defect associated with the IL-2R itself, because the T cells of both HIV-negative and HIV-positive patients could express IL-2R on stimulation with phytohemagglutinin (PHA) in vitro (data not shown). By contrast, in patients with PBMCs undergoing apoptosis, an elevated percentage of T cells expressed HLA-DR, another activation-associated surface antigen; 5 ± 1% and 8 ± 4% (mean ± SE) of the lymphocytes of HIV-negative controls and of HIV-infected children with no apparent apoptosis of PBMCs, respectively, coexpressed CD3 and HLA-DR, but 31% ± 4% of the lymphocytes from the patients with signs of apoptosis were CD3+/HLA-DR+ (P ≤ .001 when compared with the controls, and P ≤ .005 when compared with the apoptosis-negative patients) (Table 1 and Fig 2). The activated T cells were predominantly CD8+ T cells (Table 1 and Fig 3). We next examined which cells were undergoing apoptosis. PBMC were cultured for 16 to 24 hours and subsequently analyzed by flow cytometry. In light scattergrams apoptotic and nonapoptotic cells form distinct populations (Fig 4). Expression of cell surface markers was compared between cell populations containing apoptotic and nonapoptotic cells by gating on the respective cell populations. Of all cells assessed, 15% and 64% fell into the apoptotic (region 1) and nonapoptotic (region 2) cell population, respectively (Fig 4). Within the cell population containing apoptotic cells, 34% of the cells coexpressed CD3 and HLA-DR, as opposed to 5% of the cells within the nonapoptotic cells. T cells within the apoptosis-positive cell population were predominantly of the CD8+ phenotype (CD4/CD8 ratio 0.3), whereas in the nonapoptotic cell compartment, the CD4/CD8 ratio was 0.9. We conclude that in patients with PBMCs undergoing apoptosis a marked expansion of activated T cells, mainly of the CD8+ phenotype, occurs and that this cell population is at risk to undergo activation-induced cell death. These findings are in agreement with previously published data.6,13 Preliminary longitudinal assessment of the patients with apoptosis showed persistence of increased CD8+ T-cell activation (data not shown).

Occurrence of apoptosis in HIV-infected children coincides with active EBV infection. We next approached the question why the T cells had been activated in vivo. Expansion of a CD8+/IL-2R+/HLA-DR+ lymphocyte population has been suggested to be indicative for a state of chronic activation caused by a chronically active viral infection.19 A well-studied and frequently encountered example of an agent leading to such a condition is EBV. Assessing the PBMC of three patients with acute EBV infection (infectious mononucleosis) by fluorescence-activated cell sorter (FACS) analysis for expression of T-cell activation markers and by agarose gel analysis for apoptosis, we found a high percentage of activated T cells, predominantly of the CD8+ phenotype as measured by coexpression of CD8 and HLA-DR molecules (Table 1). Less than 5% of the lymphocytes of these patients expressed IL-2R (data not shown). Apoptosis was detected after overnight culture without the addition of exogenous mitogens, but not in freshly isolated PBMC. These findings are in agreement with previous reports describing apoptosis in infectious mononucleosis19-21 and resemble those in HIV-infected children with apoptosis presented here.

We hypothesized that reactivation of a latent chronically persisting viral infection or, especially in a pediatric patient population, primoinfection with such an agent could contribute to apoptosis in HIV-infected patients. We compared the results of apoptosis and T-cell activation on the one hand with the EBV status of our patients on the other hand (Table 2), EBV serving as one example for such viruses, as it has been specifically reported to reactivate in HIV-infected patients with progressive immunodeficiency.22,23 We assessed PBMC of the 16 HIV-infected patients and seven controls for the presence of EBV genome by semiquantitative PCR (Fig 5); furthermore, tissues suspected on clinical grounds
Table 2. Relation of Apoptosis and EBV Infection

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Abbreviations: EBV, Epstein-Barr virus; EA, early antigen; EBNA, Epstein-Barr nuclear antigen; GLA, generalized lymphadenopathy; HM, hepatomegaly; HSM, hepatosplenomegaly; NHL, non-Hodgkin’s lymphoma; NT, not tested; OHL, oral hairy leukoplakia; PBMC, peripheral blood mononuclear cells; VCA, virus capsid antigen.

* Number of copies per µg DNA.
† EBV genome detected in tissue.

to harbor replicating EBV were assessed for EBV genome, too (lymphomas, tonsils in one patient with recurring tonsillar hyperplasia after tonsillectomy, tongue with oral hairy leukoplakia). As shown in Table 2, eight of the 12 children with apoptosis had increased levels of EBV genome (>30 copies) in the peripheral blood indicating substantial viral replication. In two of the remaining four patients with apoptosis, EBV genome was detected in oral hairy leukoplakia.

Fig 5. Levels of EBV in PBMC. Autoradiography of semiquantitative PCR hybridized with BC3 probe. Semiquantitative PCR analyses were performed as described in Materials and Methods. Controls: serial 10-fold dilutions of EBV DNA ranging from 300,000 to 0.3 copies of Namalwa cell line were used for quantification; B, reference cell line BJAB (EBV negative). Proband samples: lane 1 (proband no. 24, Table 2), 30,000 to 300,000 copies; lanes 3, 4, and 6 (proband no. 16, 18, 23, Table 2), 300 to 3,000 copies; lanes 5 and 7 (proband no. 15 and 20, Table 2), <3 copies; lane 2 (proband no. 11, Table 2), <3 copies.
kia and hyperplastic tonsillar tissue. By contrast, in probands without apoptosis, either controls or HIV-infected children, no or only low levels of EBV genome (≤30 copies) could be detected. Five of the 10 patients with apoptosis and detectable EBV in peripheral blood and/or tissue showed markedly elevated IgG antibody titers to EBV (Table 2). In two of these five patients, IgM to VCA was detected. The clinical findings of the patients are listed in Table 2.

DISCUSSION

We and others have shown that apoptosis of PBMC in HIV-infected subjects can be observed only after the cells have been cultured.\textsuperscript{2,9,10} This finding may reflect that apoptotic cells are rapidly cleared by the reticulo-endothelial system in vivo, whereas in vitro apoptotic cells would accumulate and thus become detectable.\textsuperscript{74} The alternative hypothesis, that cells may only die on culture, seems unlikely, as lymphocytes in a preapoptotic state can be demonstrated in freshly isolated peripheral lymphocytes.\textsuperscript{11}

Apoptosis has recently been debated as potential cause of T cell depletion during HIV infection.\textsuperscript{7} Reports describing apoptosis as a consequence of in vitro activation of HIV-infected PBMC have led to the hypothesis that HIV itself might predispose PBMC to die rather than to proliferate on stimulation. However, we and others\textsuperscript{2,11} have shown that apoptosis occurs without stimulation in vitro. This, and the fact that neither in the present nor in previous studies, all HIV-infected patients showed apoptosis makes HIV as the sole trigger of apoptosis unlikely. Our observation that the occurrence of apoptosis was correlated to expansion of CD8\textsuperscript{+}/IL-2R\textsuperscript{+}/HLA-DR\textsuperscript{+} T cells (Fig 3) suggests that factors additional to HIV and able to induce such T-cell activation are involved in the process of triggering apoptosis; the lack of expression of IL-2R may contribute to apoptosis by hindering adequate T-cell activation.

Herpes viruses and, within this group of agents, especially EBV are known to be potent inducers of expansion and activation of CD8\textsuperscript{+} T cells.\textsuperscript{9,20} Furthermore, HIV-negative individuals with primary EBV infection show, beside expansion of CD8\textsuperscript{+}/IL-2R\textsuperscript{+}/HLA-DR\textsuperscript{+}, cells, also apoptosis (our results and Tomkinson et al\textsuperscript{10} and Uehara et al\textsuperscript{20}). We, therefore, wondered whether herpes viruses such as EBV, which after primary infection are not cleared from the body and, due to defective regulation, may reactivate in HIV-infected individuals,\textsuperscript{22,25,26} are relevant for the occurrence of apoptosis in HIV-infected individuals. We choose to assess the presence of EBV genome in PBMC and specific tissues as a parameter for active viral replication.\textsuperscript{22,27} Our observation of apoptosis occurring in all of the 10 patients in whom we detected increased levels of EBV genome in peripheral blood and/or in EBV-related lesions indicating increased viral replication, suggests that active EBV may be one potential trigger of apoptosis. Because HIV-infected individuals with EBV-related disorders also tend to have elevated antibody responses to EBV,\textsuperscript{28-31} the magnitude of the serologic response to EBV observed in six of the 10 patients with detectable EBV genome in blood and/or tissue further supports the activity of EBV infection. Our microbiologic investigations were limited to EBV. Thus, the potential role of other herpes viruses or other agents for the triggering of apoptosis remains to be evaluated.

Most adults have already contracted EBV infection when they acquire HIV. By contrast, vertical HIV infection of children occurs before primary infection with EBV. In vertically HIV-infected children, manifestation of clinical symptoms shows a bimodal distribution. One patient population suffers from an early onset of disease, usually within the first year of life, whereas another patient population exhibits a slowly progressive disease, comparable to that in adults.\textsuperscript{32,33}

It is tempting to speculate that the time of primary infection and/or subsequent reactivation of latent infection with EBV in the immunocompromised host may be one determinant of the clinical course in vertically HIV-infected children by triggering apoptosis.

Apoptosis of T cells was also found in macaques infected with the simian immunodeficiency virus, but not in HIV-infected chimpanzees, paralleled by the development of clinical illness in the first, but not in the latter.\textsuperscript{7} It would be interesting to investigate whether chimpanzees can be productively infected with EBV, or whether the EBV-related herpes virus pan, which has been found in chimpanzees, induces apoptosis in the T cells of these animals.

Finally, two facts may question the relevance of cell death by apoptosis for T-cell depletion and loss of immunocompetence in the acquired immune deficiency syndrome (AIDS). First, in this and previous studies,\textsuperscript{13} activation and activation-induced cell death predominantly affected CD8\textsuperscript{+} T cells, whereas progression to AIDS is commonly associated with loss of CD4\textsuperscript{+} T cells. Second, in HIV-negative individuals, T-cell death by apoptosis as occurring in primary EBV infection does not induce long-lasting T-cell lymphopenia; additional HIV-associated factors would have to be postulated. Further studies are needed to evaluate the contribution of herpes viral-triggered apoptosis to the T-cell loss leading to AIDS.

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T-cell death by apoptosis in vertically human immunodeficiency virus-infected children coincides with expansion of CD8+/interleukin-2 receptor-/HLA-DR+ T cells: sign of a possible role for herpes viruses as cofactors?

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