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HIV-1 upregulates Fas ligand expression in CD4$^+$ T cells *in vitro* and *in vivo*: association with Fas-mediated apoptosis and modulation by aurintricarboxylic acid

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SUMMARY

CD4$^+$ T-lymphocyte apoptosis has been associated with human immunodeficiency virus (HIV)-1 infection *in vitro*, paralleling the expression of Fas (APO-1, CD95) on peripheral blood mononuclear cells from patients with HIV disease. However, the link between Fas induction, T-cell activation, and cell death is unclear. We document, for the first time, marked upregulation of expression of mRNA for the ligand for Fas in peripheral blood mononuclear cells from HIV seropositive individuals, and demonstrate the ability of HIV infection to induce such expression in CD4$^+$ T cells *in vitro*. We also define the relevance of this expression to HIV-mediated CD4$^+$ T cell death. Our ability to downregulate Fas ligand message and suppress HIV-mediated apoptosis with aurintricarboxylic acid, a clinically used protease inhibitor with known activity against programmed cell death in other systems, may open up a new area of therapy for HIV infection.

INTRODUCTION

Apoptosis of CD4$^+$ T lymphocytes from human immunodeficiency virus (HIV)-1+ patients occurs spontaneously *in vitro*.[1] Suggestive evidence that it is important *in vivo* comes from four types of data: it is more frequent among peripheral CD4$^+$ T cells from rapid progressors to acquired immune deficiency syndrome (AIDS) than from long-term non-progressors;[2] high affinity anti-DNA and anti-histone antibodies, presumably induced by fragmented chromatin from apoptotic cells, occur in advanced HIV disease;[3] accelerated apoptosis is not seen in lymphocytes from HIV-1 infected chimpanzees, which generally do not develop the immunological and clinical sequelae of AIDS, while it is prominent in simian immunodeficiency virus-infected macaques with simian AIDS;[2] and elevated levels of T-cell apoptosis have been observed in lymph nodes of HIV+ patients.[4]

The underlying pathophysiology of HIV-associated apoptosis is unclear. Recently, Fas (APO-1, CD95), a membrane molecule with homology to the tumour necrosis factor-α receptor family,[5] has been implicated in its induction in transformed cells[6] and in peripheral blood mononuclear cells (PBMC).[7] Fas is also increased in freshly isolated cells from many HIV-1+ children and adults,[8,9] and HIV infected CD4$^+$ T-cell lines rapidly undergo apoptosis following exposure to anti-Fas monoclonal antibodies (mAb) in the solid phase.[6] However, the ligand for Fas, Fas-L,[10] has not been assessed in the context of HIV infection. We now show, for the first time, that Fas and FasL are both elevated on CD4$^+$ T cells in HIV disease and that, in contrast to resting or activated normal T cells,[11] HIV-infected FasL+ CD4$^+$ T cells are sensitive to rapid induction of cell death, a process susceptible to pharmacological intervention.

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MATERIALS AND METHODS

PBMC were obtained from heparinized venous blood of eight HIV seropositive patients at various stages of HIV infection (CD4$^+$ T-cell counts <50 to $>1000/mm^3$) and 10 HIV seronegative asymptomatic controls. Fas and FasL determinations were performed on PBMCs depleted of adherent cells, as well as purified total T cells and CD4$^+$ T cells isolated by magnetic Dynal bead/anti-Leu-3a tagging, all prepared as previously described.[12] Expression of Fas and FasL was quantitated by reverse transcriptase–polymerase chain reaction (RT–PCR), utilizing primers capable of amplifying a segment from nucleotides 271 to 820 of Fas cDNA,[5] or nucleotides 392 and 1182 of FasL cDNA.[11] These primers are: Fas 1: 5′-CAAGTGACTGACATCAACTCC; Fas 2: 5′-CCTTGGTTTTCCTTTCTGTGC; FasL: No. 13203: 5′-CAGCTCTTCCACCTACAG; No. 13206: 5′-TCATGCTTCTCCCTCTTCACATGG. Briefly, total cellular RNAs were first isolated from 2×10^6 cells/sample by the TriZOL (Gibco-BRL, Gaithersburg, MD, USA) method.[11] RNAs were treated with RNase-free DNase, then reverse transcribed into cDNAs, using a murine leukaemia virus reverse transcriptase (Gibco-BRL).

Aliquots of the cDNAs were then amplified by PCR, as described by our laboratory,[12] using Fas, FasL, and β-actin control (sense: 5′-TGACGGGGTCACCCACACTGTGCCCATCTA; antisense: 5′-CTAGAAGCATTTGCGGTGGACGATGGAGGG; Strategene, La Jolla, CA, USA) primers. Fifty microlitres of reaction volume, including 1× reaction buffer (Perkin Elmer, Foster City, CA, USA), optimized concentrations of $MgCl_2$, dNTP, and primers, and two units of Taq polymerase were used per reaction. The cDNAs were denatured for 2 min at 97° prior to 35 runs in a thermal cycler, with denaturation at 94° for 1 min, annealing at 55° for 1 min, and extension at 72° for 1 min in each cycle. A final extension at 72° for 5 min was included. PCR products were separated by electrophoresis in a 1·4% agarose gel, visualized by ethidium bromide staining under ultraviolet illumination, and photographed. The expected sizes of the amplicons were: Fas, 549 nucleotides; FasL, 790; and β-actin, 661.

To assess apoptosis, PBMC were plated at $0{\cdot}5 \times 10^6$ viable cells/ml in culture medium alone (RPMI-1640 plus 10% fetal bovine serum), or with various reagents, for 12–120 hr. Cells were harvested and labelled with anti-CD4 mAb Leu-3a and fluorescein isothiocyanate-conjugated goat-anti-mouse immunoglobulin. They were then fixed in 70% cold ethanol, incubated for 20 min at 4° with propidium iodide (50 μg/ml) in the presence of RNase A (300 U/ml),[13] and analysed in a cytofluorograph. Apoptosis was recognized in viable, fluorescence-positive cells by two flow cytometric methods: detection of depressed forward scatter and increased orthogonal (side) scatter characteristic of apoptotic cells,[14] and DNA histogram generation with calculation of an A_0 peak, the area of low DNA staining pre-G_1/S[1,13]. At least 5000 cells were assessed, and percent of cells in A_0 defined by computer software (MCycle Av) provided by Coulter (Hialeah, FL, USA).

Parallel cell aliquots were analysed for fragmentation of genomic DNA. Low molecular weight DNA was prepared by lysis of $2–4 \times 10^6$ cells per condition in 0·4 ml of lysis buffer, consisting of 10 mM Tris–HCl (pH 7·5), 1 mM EDTA (ethylenediaminetetraacetic acid), and 0·2% Triton X-100, followed by centrifugation and precipitation of the supernatant with 0·1 ml 5 M NaCl and 0·5 ml isopropanol overnight at −20°.[15] Samples were pelleted, washed with 70% ethanol, and air dried. The DNA was resuspended in Tris–EDTA buffer and treated with RNase A (50 μg/ml) for 1 hr at 37°. Samples were electrophoresed through 1% agarose gels at 50 V/25 mA for 3 hr, with bands visualized by ethidium bromide (0·5 μg/ml) staining.

Acute HIV-1 infection was performed using $CD4^+$ H9 and Jurkat T lymphoblasts, or peripheral blood mononuclear cell (PBMC) obtained from HIV-seronegative donors and activated with phytohemagglutinin (PHA) (2 μg/ml) for 72 hr, as targets. HIV-1 isolate IIIB at a multiplicity of infection of 0·15 was used, and HIV activity followed by assay for HIV p24 core (Gag) antigen by enzyme-linked immunosorbent assay (ELISA) antigen capture (Abbott Labs, Abbott Park, IL) of Triton-solubilized culture supernatants. Details of infection and its assessment has been described previously.[13]

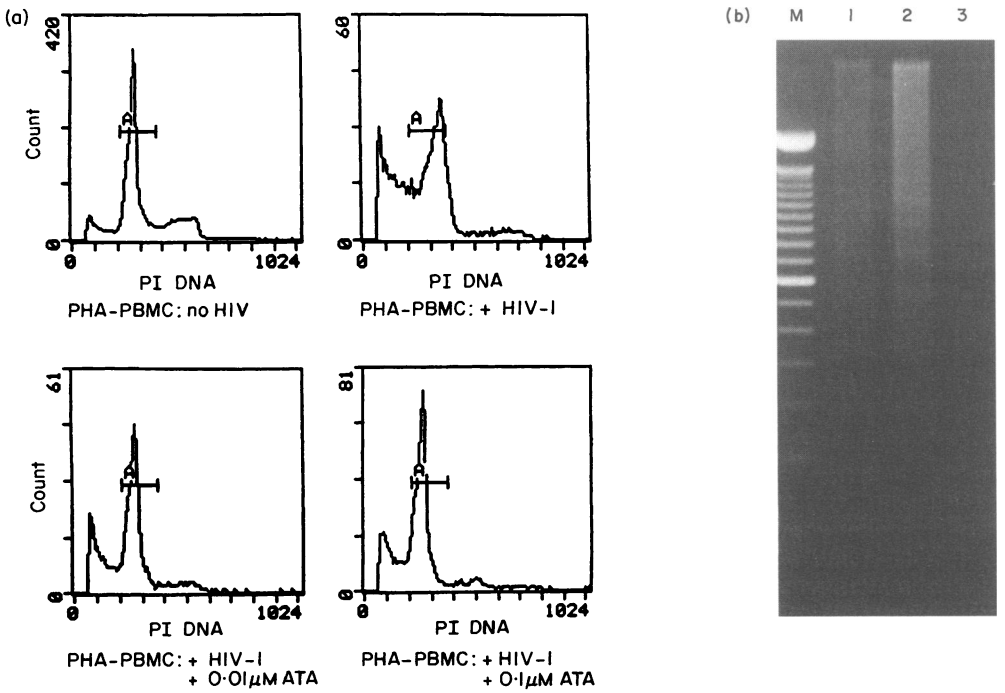

Figure 1. Demonstration of HIV-mediated apoptosis in $CD4^+$ T cells. (a) Flow cytometry. PHA-activated PBMC from healthy, HIV seronegative donors were infected with HIV-1 and cultured for 14 days in the presence of interleukin-2 (IL-2) (30 U/ml) alone or IL-2 plus ATA. Fresh culture medium plus drug was added every 3–4 days. Cells were harvested, stained for CD4 with an anti-CD4 mAb plus FITC-goat anti-mouse immunoglobulin G (IgG), fixed, and the DNA labelled with propidium iodide in the presence of RNase. Apoptosis is represented by A_0, defined by a standard computer program[13] as the area just prior to G_1/S ('A'). In this experiment it comprises 2·3% of the cell cycle in the absence of HIV, 43% with HIV + IL-2, 10% with HIV + IL-2 + 0·01 μM ATA, and 18% with HIV + IL-2 + 0·1 μM ATA. (b) DNA gel. Low molecular weight DNAs were extracted from 2×10^6 Jurkat $CD4^+$ T cells on day 7 of culture. Lane 1, mock infected; lane 2, HIV-infected; lane 3, HIV-infected, followed by addition of 0·1 μM ATA.

RESULTS

We first confirmed the finding that $CD4^+$ T cells are susceptible to HIV-mediated apoptosis. $CD4^+$ T lymphocytes in PBMC derived from five of seven HIV+ patients tested exhibited pronounced apoptosis by the criteria of low DNA staining, represented by a pre-G_1 A_0 peak that occupied from 18% to > 40% of the DNA histogram, at 18 hr of cell culture (Table 1). In contrast, all of ten HIV seronegative controls, including two with stable $CD4^+$ T cell lymphopenia, had apoptotic peaks of < 10%. These low values are consistent with additional control patients examined in our laboratory.[16] All HIV+ individuals save for patient No. 6, who was co-infected with human T cell lymphotropic virus type II, had a history of opportunistic infections and/or CD4 counts < 200 mm^3. At least 12 hours of culture was required to detect an A_0 peak.

We also established that cytopathology in $CD4^+$ T cells directly infected with HIV-1 is apoptotic by three criteria: appearance of a prominent A_0 peak on propidium iodide staining of PBMC infected with HIV *in vitro* (Fig. 1a), and depression of forward light scatter (not shown), as recognized by cell cycle analysis of fluorescence-labelled $CD4^+$ T cells; and DNA fragmentation, documented by electrophoresis of DNA extracted from HIV-infected $CD4^+$ Jurkat T cells (Fig. 1b). In the latter, oligonucleosomal bands with molecular sizes of approximate multiples of 180 nucleotides are apparent. They were noted as early as seven days after HIV infection and reached a peak at day 14.

Fas expression was undetectable (Fig. 2a, two of six controls shown) or low (four of six controls; data not shown) in unstimulated, adherent cell-depleted PBMC samples from HIV seronegative individuals. In contrast, Fas was expressed at high levels in unstimulated cells from all of eight HIV+ patients evaluated, despite equivalent levels of β-actin expression in these samples (Fig. 2b). Two Fas transcripts, the larger of anticipated size and the smaller, confirmed as a Fas product by southern blotting (not shown), and representing the transmembrane domain-truncated form of Fas,[17] were noted in most HIV+ PBMC samples (Fig. 2b). Direct HIV-1 infection of unstimulated or PHA-activated PBMC or H9 cells had no impact on Fas induction or expression (not shown).

Table 1. Spontaneous apoptosis in peripheral blood mononuclear cells from HIV-1 seropositive individuals

Patient code	Clinical status	$CD4^+$ T cell count (per mm^3)	Apoptosis untreated	(%A_0 peak) + ATA
1	AIDS	< 100	35·0	ND*
2	AIDS	< 100	30·9	4·8
3	AIDS	60	44·0	14·0
4	AIDS	19	0	0
5	Asymptomatic	152	18·0	ND
6	Asymptomatic†	1100	43·5	0
7	AIDS	181	0	0
8	AIDS	50	ND	ND

A_0 peaks were quantitated after 18 hr of cell culture as a percentage of the total DNA histogram of $CD4^+$ T cells in PBMC populations. ATA, 0·1 μM, was present for the entire culture period. Ten HIV seronegative control individuals gave < 10% A_0.

* Not done.

† Co-infected with HIV-1 and human T cell lymphotropic virus type II (HTLV-II).

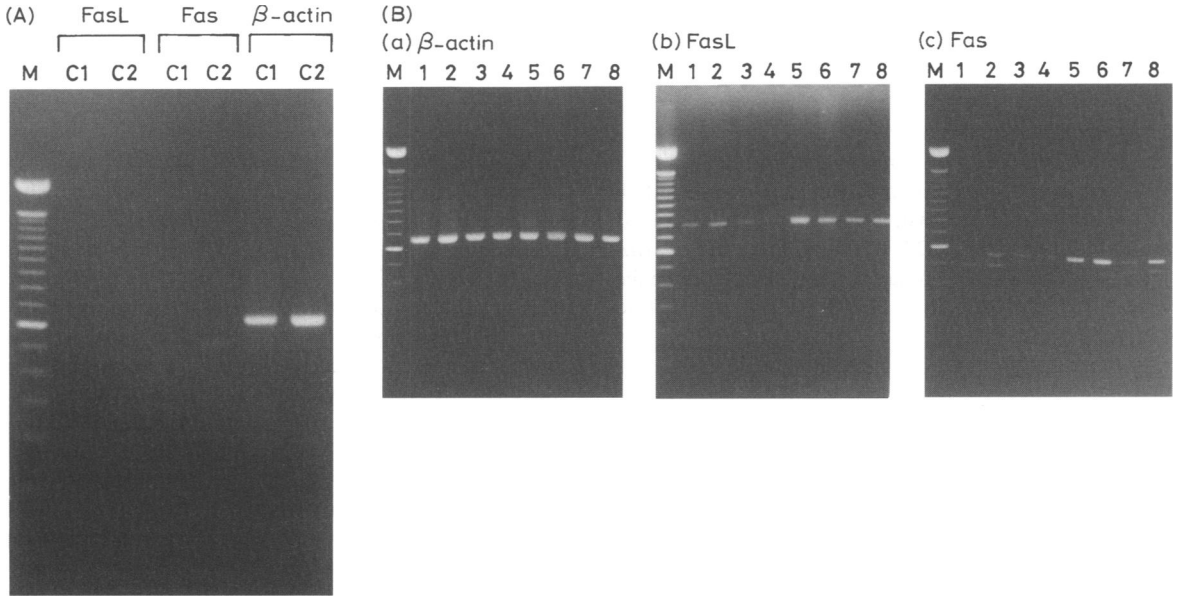

Figure 2. RT–PCR for expression of Fas and Fas ligand in control and HIV-1 + donors. (A) HIV seronegative controls. RNAs derived from 2 × 10^6 PBMC obtained from two HIV seronegative donors (C1 and C2) were reversed transcribed into cDNAs and the products amplified by PCR, utilizing β-actin, Fas, and FasL primers, as described in the text. (B) HIV+ patients. RNAs derived from 2 × 10^6 PBMC obtained from eight HIV seropositive donors at varying clinical stages of HIV disease, as outlined in Table 1, were reversed transcribed into cDNAs and the products amplified by PCR.

RT–PCR amplification of FasL mRNA in unstimulated PBMC from HIV− and HIV+ donors was also performed. No or very weak signals were seen in adherent cell-depleted PBMC from normal donors (Fig. 2a). In contrast, intense FasL signals were seen in all of eight HIV+ patients tested (Fig. 2b). In addition, in marked contrast to the lack of effect of Fas expression, infection of $CD4^+$ H9 T cells with HIV-1 led to a marked upregulation of FasL expression (Fig. 3, compare lanes 1–3).

We also examined the susceptibility of HIV-linked apoptosis to monomeric aurintricarboxylic acid (ATA), an inhibitor of two major pathways for programmed cell death, cysteine proteases, and Ca^{2+}-dependent endonucleases that cleave host chromatin in the nucleosomal linker regions.[18] $CD4^+$ T cells in PBMC from all of three HIV+ patients tested had a $\geq 60\%$ decrease in apoptotic index in the presence of ATA, with suppression of the A_0 peak (Table 1). Together with interleukin 2 (IL-2), concentrations of ATA as low as 0·01 μM suppressed A_0 by >40%, with maximal effect at 0·1 μM (60–90% inhibition).

These results were confirmed by experiments with $CD4^+$ T cells directly infected with HIV. ATA suppressed the A_0 peak (Fig. 1a) and DNA fragmentation (Fig. 1b) associated with HIV infection. In addition, the ability of PBMC infected with HIV to mount a proliferative response following re-exposure to mitogen, as assessed by ^{3}H-thymidine incorporation, was preserved in the presence of ATA, despite the fact that ATA had no impact on the amount of HIV produced in these cultures. Thus, PHA restimulation of activated PBMCs maintained in culture for 14 days in the absence of HIV gave a stimulation index of 6·1–6·3, which fell to 1–1·6 in HIV inoculated cultures. This index rose to 3·9–6·0 in the presence of 0·01 μM ATA, while HIV activity was between 106–112 ng/ml in all cultures. This latter result is important for two reasons. First, much higher levels of ATA (3–100 μM) can non-specifically inhibit HIV replication, presumably through its ability to block a variety of enzymes, including proteases, and reverse transcriptases,[18] critical to HIV replication, but are also highly cytotoxic. Second, inhibition of apoptosis without preservation of at least some T-cell function would not be of much therapeutic utility.

ATA had no impact on Fas expression in HIV-infected H9 cells (data not shown). In contrast, it markedly decreased FasL expression in immortalized $CD4^+$ H9 cells infected *in vitro* (Fig. 3, lanes 1–4), and had a similar although less potent effect on FasL expressed by cells from an HIV+ patient (Fig. 3, lanes 5 and 6).

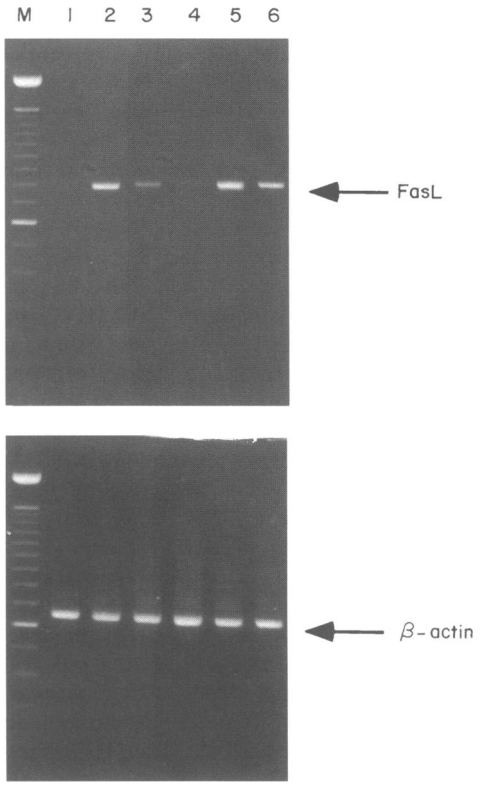

Figure 3. Effect of aurintricarboxylic acid on FasL expression in the setting of HIV infection. $CD4^+$ H9 T cells were infected with HIV-1 in the presence and absence of ATA (0·1 μM). RNAs were prepared from 2×10^6 cells at various times following infection and FasL identified by RT–PCR, as described in the legend to Fig. 2. RNAs prepared from PBMC of HIV+ Patient 6 (Table 1) were similarly evaluated following a four-day exposure to buffer or ATA (0·1 μM). Lane 1: H9; lane 2: H9 + HIV, 4 days post-infection; lane 3: H9 + HIV, 14 days post-infection; lane 4: H9 + HIV + ATA, 14 days post-infection; lane 5: HIV + Patient 6 PBMC, 4 days of culture; lane 6: HIV + Patient 6 PBMC + ATA, 4 days culture.

DISCUSSION

Our experiments establish a link between the accelerated apoptotic death of T lymphocytes seen in HIV disease and enhanced expression of both Fas, as reported here and by others,[8,9] and Fas ligand. T cells, when activated during many viral infections, may be primed to undergo apoptosis on antigen receptor stimulation via FasL-mediated cross-linking of Fas, involving membrane-associated or soluble forms of the FasL molecule.[19] This contrasts with HIV infection, however, where progressive loss of $CD4^+$ T cells occurs accompanied, as demonstrated here, by FasL expression at both high and low levels of peripheral $CD4^+$ T cells (Table 1). Although the mechanism for FasL upregulation by HIV is unclear, it has recently been shown that the HIV-1 envelope glycoprotein gp120 as well as its regulatory protein Tat, which can be released from infected cells and taken up via integrin receptors, can both induce FasL *in vitro*.[20]

It should be noted that the necessity for direct HIV infection of $CD4^+$ T cells in order to elicit apoptosis *in vivo* is in question, as is the specificity of HIV-associated apoptosis for the $CD4^+$ T lymphocyte subset.[2,4] Indeed, elevated membrane expression of Fas has been demonstrated on both $CD4^+$ and $CD8^+$ T cells from HIV+ patients,[21] while it is predominantly the former that are depleted in HIV/AIDS. Accelerated death of the $CD8^+$ subset may relate to 'fratricidal' lysis, by which $CD4^+$ T cells induced to undergo apoptosis can induce an apoptotic death in adjacent $CD8^+$ T cells. HIV infection of the $CD4^+$ subset would then limit its replacement, even as the $CD8^+$ T-cell subpopulation could be persistently restored.

The exact process by which ATA blocked apoptosis in $CD4^+$ T cells infected with HIV *in vitro* and in such cells from HIV+ patients, is unknown. ATA suppresses one activity integral to DNA cleavage in apoptosis, Ca^{2+}-mediated

endonuclease activation, but ATA is also a potent anti-apoptotic agent in anucleate T cells.[22] Fas-mediated apoptosis in T lymphocytes also requires multiple interleukin 1β converting enzyme-like proteases,[23] which could be blocked by the anti-protease activity of ATA. In addition, we have shown that ATA suppressed HIV-associated upregulation of FasL. This may be analogous to the effect of retinoic acid and glucocorticoids, which inhibit programmed cell death in murine T cells in parallel with their ability to block upregulation of FasL.[24] Differential levels of expression of Fas/FasL, or involvement of other pathways, may also be involved in HIV-associated apoptosis, as two of the seven patients tested had enhanced expression of Fas and its ligand (Fig. 2), yet did not show apoptosis *in vitro* (Table 1). Our experiments do suggest that interference with FasL activity in some cell populations may represent a new therapeutic modality to block Fas-associated cell death in HIV infection, and perhaps other disorders. In this regard, ATA has already been tested clinically, and in animals, as a protease inhibitor capable of inhibiting certain thrombotic processes,[25] which are also common in HIV disease.[26]

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