CONCISE COMMUNICATION

Low Levels of Perforin Expression in CD8+ T Lymphocyte Granules in Lymphoid Tissue during Acute Human Immunodeficiency Virus Type 1 Infection

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Human immunodeficiency virus (HIV)–specific cytotoxic T lymphocyte (CTL) responses are detectable shortly after the acute phase of HIV infection, but they cannot control viral replication and prevent development of chronic immune suppression. This article describes a defect in the coexpression of perforin in granzyme A–positive CD8+ T cells in lymphoid tissue from patients with acute HIV infection and a reduction in the perforin-dependent nuclear translocation of granzyme A. Furthermore, intracellular levels of HIV DNA and RNA found in lymphoid tissue were higher (10–100 times) than those found in blood, and blood samples showed more-coordinated cellular perforin/granzyme A expression. This suggests that mechanisms inhibiting CTL-mediated cytotoxicity are operative in lymphoid tissue early in the course of HIV infection.

Acute human immunodeficiency virus (HIV) infection (aHI) is characterized by an exponential increase in viremia to peak levels of as much as 10^9 copies/mL of plasma. Virus levels decrease after the first weeks of infection and reach a viral set point within the next 3–6 months [1]. The concomitant detection of novel HIV-1–specific CD8+ cytotoxic T lymphocyte (CTL) responses in the peripheral blood soon after the initial peak of viremia [2] and the inverse correlation between HIV-specific precursor CTL frequency and plasma viral RNA load [3] suggest that the CD8 cytotoxic response may be responsible for the acute fall in viremia and the subsequent partial control of viral replication that occur [4]. In addition, selective depletion of CD8+ lymphocytes in simian immunodeficiency virus–infected rhesus monkeys was shown to result in a rapid increase in viremia, which supports the suggestion that CD8+ T cells have a role in control of viremia [5]. CD8+ T cells may, however, also operate through noncytotoxic mechanisms mediated by factors such as cell antiviral factor and β-chemokines (macrophage-inhibiting protein–1α, macrophage-inhibiting protein–1β, and RANTES) [6]. The different effector functions exerted by CD8+ T cells, however, ultimately fail to control viral replication.

Granule exocytosis, one of the mechanisms for CTL cytolysis, is mediated by secretory lysosomes containing the pore-forming protein perforin and serine proteases termed "granzymes" [7]. The combined exocytosis of perforin and granzymes is antigen specific, major histocompatibility complex class I restricted, and calcium dependent [7]. The other CTL-mediated mechanism operates through the ligation of Fas receptors by Fas ligand (FasL) located on effector cells. Recent findings suggest that the perforin/granzyme A–dependent pathway is a key mechanism contributing to the specific elimination of HIV-1–infected cells [8] and that Fas/FasL interactions may generate bystander cell death in HIV infection [9].

Mutant mice that lack either functional Fas or FasL have normal susceptibility to viral pathogens and exert intrinsic NK and CTL functions. However, these animals develop massive lymph-

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Patients gave written informed consent prior to the sampling of lymphoid tissues and blood. All procedures were reviewed by the institutional review boards and ethical committees of each participating site. The experimentation guidelines of the Karolinska Institutet, Huddinge, Sweden, were followed in conducting the clinical research.

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adenopathy and fatal autoimmune disease. Perforin-deficient mice are, on the other hand, characterized by increased susceptibility to viral pathogens (e.g., lymphocytic choriomeningitis virus), defective alloreactive reactions, and the absence of NK cell cytolytic function, as well as reduced surveillance against tumors [10].

A defect in the production or expression of cytolytic effector molecules in T and NK cells involved in the killing of infected cells could result in persistence of replicating HIV cells. Indeed, skewed maturation of memory HIV-1–specific $CD8^+$ T cells has recently been described [11]. We report on our study of the expression of perforin and granzyme A in lymphoid tissue (LT), where the bulk of HIV replication occurs.

Subjects and Methods

Study cohort. Twenty-four patients from the international (Australia, Europe, and Canada) QUEST study (protocol GW PROB 3005) participated. Patients were eligible for study entry if they had p24 antigenemia or HIV viremia and if ≤3 bands were seen on Western blot analysis of serum. Of the 24 eligible patients, 22 were HIV negative at enrollment (all of whom seroconverted within 30 days after study entry), as determined by third-generation ELISA. All subjects had acute viral syndrome before study entry, with symptoms lasting 1–28 days (mean, 11 days). Sixteen of the 24 patients had never received highly active antiretroviral therapy, and 8 had received such therapy for 1–7 days. At baseline, patients had $CD4^+$ cell counts of 280–1380 cells/μL of blood (mean, 604 cells/μL) and $CD8^+$ cell counts of 480–1190 cells/μL of blood (mean, 620 cells/μL), and plasma HIV RNA loads ranged from 5.4×10^4 to 4.8×10^6 copies/mL (mean, 650,000 copies/mL). Peripheral blood mononuclear cells (PBMC) from 10 of the 24 aHI patients were obtained by random selection before the initiation of antiretroviral therapy. Tonsil biopsy and PBMC samples were obtained from 6 patients with symptomatic acute Epstein-Barr virus mononucleosis (aEBV) and from 15 HIV-seronegative adults with tonsil hyperplasia (control subjects). Colon biopsy samples were also obtained from 6 healthy individuals.

Perforin and granzyme A assessment. Cryopreserved, 8 μm–thick LT and colon biopsy samples were stained for perforin and granzyme A as described elsewhere [12]. Phenotypic staining was done for CD3, CD4, CD8, and CD56. Acquired computerized image analysis was used for quantification of the stainings [12].

Perforin in situ detection. An in situ hybridization method, with a cocktail of 4 short, nonoverlapping oligonucleotide probes representing the fourth exon of perforin, was used. Probes based on oligonucleotide sequences were labeled with S^{35} synthetic oligonucleotide probes (Scandinavian Gene Synthesis). A probe complementary to the antisense strand of a human perforin probe was used as a control. Each section was examined by dark-field microscopy. The cutoff level for positive cells was >25 grains/cell, as measured by in situ imaging with the Quantimet Q 550 IW (Leica Cambridge Instruments).

Intracellular virus load. Cell-associated HIV-1 RNA and DNA were measured in the same cell aliquot, using the reagents from the Amplicor HIV-1 Monitor assay (Roche). For cell-associated HIV-1 DNA quantitation, the nucleic acid preparation was incubated with DNase-free RNase A (Sigma). The specimen and the DNA preparation were added to master mix buffer containing 25 copies of DNA IQS (Roche) and then amplified. The level of detection was ~3 copies/10^6 cells for RNA and ~5 copies/10^6 cells for DNA.

Results

The frequency of $CD8^+$ T cells (22%–31% of total cells) was up-regulated 4–9-fold, and the frequency of granzyme A–positive cells (9%–13% of total cells) was up-regulated 10–100-fold in tonsil, lymph node, and colon biopsy samples from aHI patients and in LT from aEBV patients, compared with HIV-seronegative controls ($P < .001$). Granzyme A–expressing cells were located predominantly in the parafollicular area of the lymph nodes. The incidence of perforin-expressing cells (0.1%–1.5%) in patients was not statistically significantly higher than in control subjects ($P = .56$; $P < .05$ was considered to be significant); however, it was significantly lower in aHI patients than in aEBV patients ($P < .01$) (figure 1*A* and 1*B*). Coexpression of granzyme A and perforin is thought to be required for effective $CD8^+$ T cell–mediated cytotoxic responses against virally infected cells [7]. A granular colocalization of perforin and granzyme A was detected in the same intracellular vesicles in HIV- and EBV-infected LT (figure 1*A* and 1*B*). In total, ≤10% of the granzyme A–positive cells coexpressed perforin in the LT from aHI patients, whereas the great majority of granular granzyme A–expressing cells from aEBV patients contained perforin (figure 1*A* and 1*B*). The frequency of granzyme A–positive cells in uninfected tissues from control subjects varied from 0.1% to 0.5%, whereas perforin expression varied from 0.01% to 0.1%.

Two-color staining both for perforin/CD8 and granzyme A/CD8 revealed that >90% of perforin- and granular cytoplasmic granzyme A–expressing cells in LT belonged to the $CD8^+$ T cell lineage (figure 1*C*). The frequency of $CD56^+CD3^-$ NK cells in LT from aHI patients was <1% of total cells; hence, these cells did not significantly contribute to granzyme A and/or perforin expression (data not shown).

In situ imaging of cells hybridized for perforin mRNA in LT from aHI patients showed a low frequency of perforin-expressing cells, which was consistent with results for protein expression (figure 2). A significant increase in perforin mRNA–positive cells, compared with samples from aHI patients and LT from control subjects, was demonstrated by in situ hybridization in samples from aEBV patients ($P < .01$) (figure 2).

Perforin is a unique regulator of transfer of granzyme-containing endocytic vesicles to target cells. Perforin is not required for the cellular uptake of granzymes, but it is instrumental in the rapid cytoplasmic transportation of granzymes to the nucleus in target cells, in which granzymes cleave and activate procaspases [7]. In the present study, granzyme A–positive cells showed either a localized granular and membrane-associated localization or a nuclear-staining pattern. Granzyme A nuclear translocation was analyzed by combined staining of granzyme A and a nuclear-staining signal (4′4-diaminidino-2-phenylindole). We found that

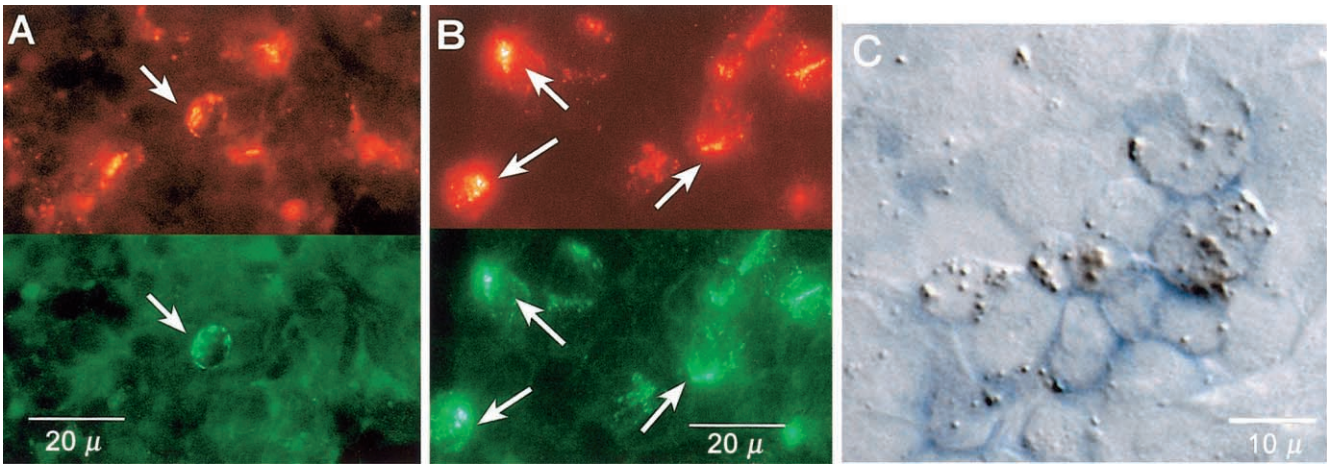

Figure 1. *A,* Two-color confocal immunofluorescent staining of granular granzyme A–positive (red Alexa 546 [Sigma]; *upper panel*) and perforin mRNA-expressing (green Alexa 488 [Sigma]; *lower panel*) cells *(arrows)* in a lymphoid tissue (LT) biopsy sample from a patient with acute human immunodeficiency virus infection shows a significant increase in the no. of cells with granular expression of granzyme A in LT. *B,* The same stains performed on LT from a patient with acute Epstein-Barr virus mononucleosis show massive up-regulation of colocalized granzyme A *(upper panel; arrows)* and perforin expression *(lower panel; arrows). C,* Immunohistochemical 2-color staining of granzyme A–positive cells (brown 3′3-diaminobenzidine [Sigma]) and $CD8^+$ cells (blue alkaline phosphatase [Sigma]) in an LT biopsy sample from a patient with acute human immunodeficiency virus infection. More than 90% of all granzyme A–positive cells were $CD8^+$ T cells.

only a small fraction ($\leq$10%) of all granzyme A–positive cells in LT from aHI patients were localized in the nucleus, and nuclear granzyme A translocation was much more prevalent in aEBV patients ($\leq$50% of total granzyme A–positive cells).

Perforin-expressing cells are rarely detected in secondary LT in the absence of ongoing immune activation [12]. However, PBMC obtained from healthy individuals expressed both perforin and granzyme A in a substantial number of $CD8^+$ T cells, as well as in NK cells (14%–17% of all mononuclear cells). PBMC from aHI patients ($n = 10$) showed a marginal but significant increase (19%–24% of all cells) in concomitant coexpression of granzyme A and perforin, compared with PBMC from control subjects ($n = 10$) ($P < .05$). A similar pattern was observed in PBMC from aEBV patients ($n = 6$), although at significantly higher levels (28%–32% of all cells) than were observed in aHI patients ($P < .01$).

We next assessed the intracellular HIV RNA and DNA loads in the LT and PBMC samples from aHI patients, using a sensitive reverse-transcriptase polymerase chain reaction assay and a polymerase chain reaction assay to exclude the possibility that a low level of perforin expression in LT was the result of low virus load. The mean cell-associated HIV RNA and DNA loads were 4.9 log RNA copies/10^6 cells and 3.5 log DNA copies/10^6 cells in LT versus 2.9 log RNA copies/10^6 cells and 2.7 log DNA copies/10^6 cells in PBMC (table 1). Significantly higher levels of both HIV RNA and HIV DNA were found in mononuclear cells from LT than in PBMC ($P < .001$ and $P < .003$, respectively). The ratio of HIV RNA to HIV DNA in aHI patients also was higher in LT than in PBMC. As has been described elsewhere [13], these results are consistent with the presence of a statistically significantly higher number of HIV proviral DNA copies and an increased level of viral replication in mononuclear cells in lymph nodes, compared with PBMC.

Discussion

Different types of granzymes generate similar types of apoptosis-mediated activity. Consequently, the absence of one gran-

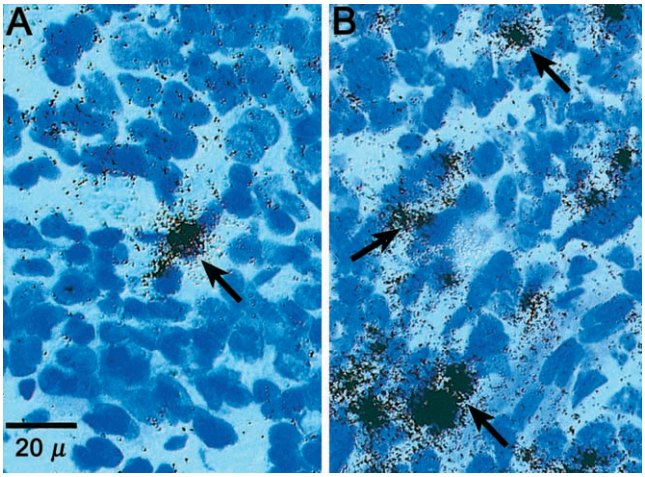

Figure 2. Photo of perforin mRNA–expressing cells in lymphoid tissue in patients with acute human immunodeficiency virus infection *(A)* or acute Epstein-Barr virus mononucleosis *(B)*. Cells expressing perforin mRNA *(black grains; arrows)* were predominately localized in the extrafollicular area. Comparison with sense probes showed that positive cells could be defined by the accumulation of >25 grains/cell. S^{35}-labeled probes (Scandinavian Gene Synthesis) were used, and cells were counterstained with cresyl violet.

Table 1. Intracellular human immunodeficiency virus (HIV) RNA and DNA loads in peripheral blood mononuclear cells and lymphomononuclear cells from 10 patients with acute HIV infection.

	Peripheral blood mononuclear cells		Lymphomononuclear cells	
Patient	RNA copies/ 10^6 cells	DNA copies/ 10^6 cells	RNA copies/ 10^6 cells	DNA copies/ 10^6 cells
1	3.14	2.74	5.78	3.48
2	3.36	2.00	4.88	4.00
3	3.14	1.57	4.30	3.98
4	3.14	2.92	5.33	3.98
5	1.23	2.48	3.78	3.02
6	3.89	3.36	5.15	3.39
7	3.29	3.27	4.38	2.79
8	3.56	2.97	5.32	3.16
9	1.54	2.66	5.70	3.11
10	3.85	3.31	4.47	3.87
Mean	2.9	2.7	4.9[a]	3.5[b]

NOTE. Reverse-transcriptase polymerase chain reaction and polymerase chain reaction were used to determine intracellular HIV RNA and DNA loads. Values are log values. Mann-Whitney calculation was performed for individual comparison of the intracellular ratio of viral RNA to viral DNA between blood and lymph nodes.

[a] $P < .001$.

[b] $P < .003$.

zyme type can be compensated for by another granzyme family member. Granzyme A, however, induces apoptosis even in cells that overexpress Bcl-2, whereas granzyme B– and C–mediated CTL activity may be inhibited by Bcl-2. This implies that granzyme A may use a novel pathway to induce cell death [14]. HIV-infected cells may up-regulate Bcl-2 and therefore can be partially protected from granzyme B and C activity, as well as from Fas/FasL-mediated immune attacks. This strengthens the role of the granzyme A/perforin pathway in CTL-mediated elimination of HIV-infected cells [8]. It could be argued that the low levels of perforin expression observed in the present study were the result of early tissue sampling, before CTL maturation occurred. However, this possibility was not supported by the observation of similarly low perforin levels in LT after 6 months of infection [12].

These data show that granzyme A was induced in conjunction with a relative lack of perforin expression in LT from aHI patients, in contrast to samples from aEBV patients, in which granzyme A and perforin were concomitantly induced. This dissociation between granzyme A and perforin expression in aHI patients may contribute to impaired CLT activity at this site. Identification of factors responsible for low perforin expression in LT may lead to the development of new therapeutic strategies aimed at restoring cytotoxic T cell function in patients with HIV infection. Furthermore, it has recently been shown that HIV tetramer–positive $CD8^+$ T cells do not express the molecules involved in efficient trafficking to LT via high endothelial venules [15]. This may have contributed to the low frequency of perforin-positive cells in the LT and in the high rates of viral replication seen at this site.

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