Perforin is not co-expressed with granzyme A within cytotoxic granules in CD8 T lymphocytes present in lymphoid tissue during chronic HIV infection

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Background: Residual HIV-1-infected cells are poorly eliminated from lymphoid tissue (LT) reservoirs by effector cytotoxic T lymphocytes (eCTL) despite antiretroviral therapy. Perforin and granzyme A (grA) constitute major effector molecules within eCTL granules that induce apoptosis and lysis of virally infected cells.

Objective: Expression of perforin and grA was studied at the single cell level in LT and blood from 16 patients infected with HIV-1 (stage A1–C) who were not taking antiretroviral therapy.

Method: Immunohistochemical analysis by in situ imaging of cells from blood and LT.

Results: Quantitative *in situ* imaging showed that perforin-expressing CD8 T cells comprised 0.3–1.5% of total cells within the LT from recent HIV-1 seroconverters, while grA was found in 2.1–7.2% of total cells. However, despite high-level grA upregulation (1.5–4.5% of total cells) compared with that in non-infected individuals (0.4–0.9%), perforin expression remained low (< 0.1% of total cells) ($P < 0.02$) in LT from patients with chronic HIV-1 infection (stage A2-C). This contrasted with findings in peripheral blood mononuclear cells (PBMC) from the same HIV-1 infected cohort where perforin was detected in 13–31% of all PBMC, which was 10- to 100-fold higher than in lymphoid tissue ($P < 0.001$); grA was found in 14–32% of total PBMC. Two-colour staining showed that granular expression of perforin and grA was restricted to CD8 T cells in over 90% of total cells in both LT and blood.

Conclusions: These findings indicate that cytotoxic perforin expression is impaired at local sites of HIV replication within lymphoid tissue. Since perforin is required together with grA for granule-mediated cytolysis, the low perforin expression in the LT may limit the ability of eCTL to eliminate HIV-1 infected cells in lymphoid tissue.

AIDS 1999, **13**:1295–1303

Keywords: HIV, cytotoxicity, CTL, perforin, granzyme A, lymphoid tissue

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Sponsorship: This work was supported by the National Cancer Institute (grant 2490), the Swedish Medical Research Council (grant 10850), the Bert von Kantzow Foundation and the National Institues for Health (grant AI41536-01).

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Received: 22 December 1998; revised: 26 April 1999; accepted: 4 May 1999.

Introduction

Cytotoxic T cell (CTL) activity against HIV-1 infection varies over the course of HIV infection [1–6]. Ultimately, effector CTL (eCTL) activity in HIV-infected individuals fails to control viral production and disease progression to AIDS [7]. Nevertheless, for a period following primary infection, numbers of eCTL appear to be correlated with reduction in HIV-1 viral load [1–3]. In asymptomatic HIV-infected patients, levels of activated CD8 T lymphocytes have been associated with HIV-1 viral load [8]; recently, tetrameric complexes of HIV-1 peptide-specific HLA class I molecules have been used to demonstrate a direct relation between the incidence of HIV-1 antigen-specific memory CTL cells in blood and plasma HIV RNA levels [9]. The eCTL lyse their targets either through antigen-specific MHC-restricted calcium-dependent exocytosis by lytic granules containing perforin, granzyme and tumor-induced attack (TIA-1) proteases or by Fas-mediated apoptosis [11–12]. Potentially, eCTL can also regulate HIV replication through non-cytolytic pathways that involve the release of chemokines complexed with proteoglycans and other soluble factor(s) including cytokines [13,14].

However, while a decrease in HIV-specific eCTL activity, in particular *gag*-specific CTL, often occurs in late-stage infection, no predictive association has been reported between levels of CD8 T cell eCTL activity at the onset of infection and ultimate disease outcome [5,15,16]. In general, neither HIV-specific eCTL nor memory CTL can prevent progression to AIDS despite their high frequencies in blood and putative anti-HIV activity *in vitro* [17]. It remains to be determined why these expanded eCTL cannot control the infection and prevent disease progression. A rapid selection of CTL-escape viral mutants may be one explanation [3]. The Ca^{2+}-dependent granule exocytosis pathway induced after specific T cell receptor engagement has been shown to be the dominant mechanism for CTL lysis of HIV-1 in infected chimpanzees [18,19]. The cytolytic granule contents, including perforin and serine proteases such as granzyme A and B (grA and grB), are released into the intercellular space between the CTL and its target [20]. Levels of these molecules are elevated in the plasma from HIV-seropositive patients, suggesting sustained peripheral CTL activation [21]. One may question if this arm of antiviral immunity is functioning within lymphoid sites since potent antiretroviral therapy administered for over 30 months was unable to eliminate HIV-1 tissue reservoirs effectively despite substantial reduction in plasma HIV RNA [22,23]. A functional block in cytolytic molecule expression in the HIV-specific eCTL lymphoid tissue (LT) *in vivo* might explain why the observed combination of active type 1 cytokine production in LT and CD8 T cell recruitment did not confer adequate eCTL activity for protective immunity. In this study, we compare expression of perforin and grA within single CD8 T cells in LT and blood from HIV-infected patients at various stages of HIV infection.

Material and methods

Subjects

Cryopreserved lymph nodes and tonsil biopsies were obtained from 16 HIV-1-infected individuals who were not receiving antiretroviral treatment. All patients gave signed informed consent. Institutional review board approval was obtained from Rush Medical School, University of Colorado and University of Degli, Rome. Peripheral blood mononuclear cells (PBMC) were obtained from seven of these patients. PBMC from an additional four HIV-1 infected patients were also included. Lymph nodes, tonsil biopsies and PBMC were obtained from five adult HIV-seronegative controls. Four tonsil biopsies were obtained from patients with acute Epstein–Barr virus (EBV)-induced infectious mononucleosis. PBMC was also obtained from an additional two EBV-infected patients and from three healthy controls.

Plasma HIV-1 viremia was measured by branch-DNA assay (Chiron, Emeryville, CA, USA). CD4 and CD8 T lymphocyte counts were performed by routine clinical laboratory testing.

Immunohistochemical staining and phenotyping

Anti-perforin (Delta G9 mAb from Endogen, Woburn, MA, USA) was used against human pore-forming protein; anti-granzyme A (protein A purified) (CB9 monoclonal antibody) was provided by Dr Judy Lieberman [24]. The following monoclonal antibodies were used for cell surface staining: CD3, CD4, CD8 and CD56 (Beckton–Dickinson, Rutherford, NJ, USA).

Adherent monocytes from HIV-seropositive and healthy blood donors were isolated from EDTA-treated blood. The staining procedure used for cyropreserved tissue and PBMC to identify cytotoxic effector molecules as well as cell surface markers at the single cell level has been described previously [23,25]. For two-colour immunohistochemcal analysis, cells stained for perforin and grA (alkaline phosphatate substrate kit, Vector Laboratories, Burlingame, California, USA) cells were incubated with a second biotinylated monoclonal antibody to identify cell surface markers (CD3, CD4, CD8, CD56) as well as isotype-specific controls IgG_1, IgG_{2b} (Dako, Glostrup, Denmark) in balanced salt solution containing 0.1% saponin and colour was developed with diaminobenzidine (Vector Laboratories). Counter-staining used haematoxylin.

Quantification of cells by acquired computerized image analysis

Digital images of stained samples were transferred from a DMR-X microscope (Leica, Wetzlar, Germany) into a computerized image analysis (ACIA) system (Quantimet 5501W, Leica, Cambridge, UK) that allowed detection of 16.7 million different colours. The total tissue area of the sectioned lymph node and tonsil biopsies comprised 8–18 mm^2. The cellularity in each section was assessed by calculating the number of nucleated cells stained by hematoxylin per total section area using densitometric and morphometric criteria set by a software program [26]. In addition, positively stained cells were identified and assessed per total number of hematoxylin-positive cells ($0.2–1.5 \times 10^5$ cells/section) present within the total tissue area in a semiquantitative way by a specialized software program [26].

Statistical analysis

Correlation between quantitative variables was evaluated by the Spearman coefficient of correlation. A logarithmic transformation of viral plasma RNA values was used in the data analysis. Two-sided Student's test was used for statistical significance of correlations; a *PT* value of less than 0.05 was considered significant.

Results

Distribution of CD8 and CD4 T cells in lymphoid tissue

LT from HIV-infected patients with CDC stage A1 [27] disease showed a significant increase (four- to six-fold) in CD8 T cells compared with the level in uninfected controls. These cells were predominantly located in the extrafollicular areas in all HIV-infected patients (stage A1–C) within both tonsils and lymph nodes and represented 7.2–41% of all cells (Table 1). Follicular destruction and CD8 T cell depletion was evident in all CDC stage C, HIV-infected patients. A negative correlation was found between CD8 T cell indices in LT and the plasma HIV level ($r = 0.58$; $P = 0.04$). The relative proportion of CD4 T cells within HIV-infected LT varied between 7.4 and 33.6% of total cell population, with the lowest values in more advanced disease (Table 1); however, CD56+CD3– natural killer cells comprised 1–3% of total cells throughout disease (data not shown). The cellularity in the biopsies varied with severity of disease. *In situ* imaging revealed that LN from healthy controls had the highest mean cellularity ($96 \pm 2\%$) followed by LN from asymptomatic stage A HIV-infected patients ($81 \pm 8\%$) and then by LN from those with late-stage disease (stage C) ($34 \pm 15\%$).

Table 1. Clinical and virological characteristics of LT and peripheral blood mononuclear cells (PBMC) in patients with HIV-1infection, primary infectious mononucleosis or tonsillar hyperplasia and in healthy controls.

Patient No. Sample	CDC class	Blood CD4 cell count ($\times 10^6$ cells/l)	Plasma HIV RNA (copies/ml)	CD4 in tissue (% total cells)[b]	CD8 in tissue (% total cells)[b]
HIV infection[a]					
1 lymph node + PBMC	A1	504	ND	29.6	30.7
2 lymph node	A1	564	ND	28.5	32.7
3 lymph node	A2	443	ND	33.6	32.3
4 lymph node	A2	464	149 049	9.3	11.2
5 lymph node	C	163	18 742	7.4	7.2
6 lymph node + PBMC[c]	A1	342	29144	27.3	19.8
7 lymph node + PBMC[c]	A1	302	107 204	28.5	15.9
8 lymph node[c]	A1	705	22 389	21.8	13.5
9 lymph node	C	50	1 253 987	16.0	9.6
10 lymph node + PBMC	A1	967	21 890	29.0	41.0
11 lymph node	C	12	327 112	16.1	19.0
12 lymph node	A1	811	5 482	10.1	15.1
13 lymph node	A2	234	78 589	21.5	26.6
14 tonsil + PBMC	A2	403	6 780	25.0	20.7
15 tonsil + PBMC	A1	878	3 770	22.2	27.6
16 tonsil + PBMC	A2	374	23 250	32.8	22.9
Controls					
Infectious mononucleosis[d]					
1 Tonsil + PBMC	2450	–	28.2	31.0	
2. Tonsil + PBMC	1775	–	17.2	23.2	
3. Tonsil + PBMC	1895	–	32.8	31.3	
4. Tonsil + PBMC	1950	–	28.5	27.3	
Tonsillar hyperplasia					
5. Tonsil, + PBMC	1230	–	17.5	8.3	
6. Tonsil + PBMC	990	–	15.0	5.7	
7. Tonsil + PBMC	1420	–	27.9	8.6	
8. Tonsil + PBMC	1080	–	23.1	3.0	
Healthy[d]					
9. Lymph node + PBMC	1100	–	16.9	3.5	

[a]Additional PBMCs were obtained from four HIV-1-infected untreated volunteers in CDC stage A1–C. [b]Phenotypes of cells was assessed by acquired computerized image analysis. [c]These patients were recent symptomatic seroconverters; biopsies were obtained 4–5 months after primary symptoms appeared. [d]PBMC were also obtained from two acute EBV infected patients and three healthy controls.

Perforin and granzyme A expression in lymphoid sites

The frequency of cells expressing perforin and grA cytotoxic effector molecules was determined by ACIA in LT from HIV-infected patients and controls (Table 1). A characteristic staining pattern of perforin- and grA-expressing cells resulting from accumulation of these proteins in cytoplasmic granules was observed in LT (Fig. 1A and 2A,B). This facilitated identification of single perforin- and grA-expressing eCTL by the ACIA technique (Fig. 3). By two-colour staining, > 90% of the perforin- or grA-expressing cells in LT were CD8 T cells (Fig. 2C). A few of the total perforin- or grA-positive cells were of the CD56+CD3– phenotype (2–4%, data not shown).

Perforin and grA were predominantly expressed in CD8 T cells in samples obtained from three recent HIV-1 seroconverters within 4–5 months from onset of symptoms of primary infection. Granular perforin expression was found in 0.3–1.5% of the total cells within LT (1.5–4% of all CD8 T cells in the tissue) while grA could be detected in 2.1–7.2% of total cells (Fig. 2D). In contrast, LT from 13 chronically infected patients in stage A1–C who were not receiving antiretroviral therapy was significantly lacking perforin-expressing cells (mean < 0.1% of total cells) ($P < 0.02\%$) (< 0.3% of all CD8 T cells) but revealed sustained grA expression (mean 3.0 ± 1.5%; range 1.5–4.5%, of total cells) (Fig. 3E).

Much higher levels of perforin- and grA-expressing cells were evident in LT from patients with acute infectious mononucleosis. CD8 perforin-expressing cells were significantly upregulated in acute infectious mononucleosis, to 5–12% of total cells, compared with biopsies from the 16 HIV-1-infected patients ($P < 0.001$) (Fig. 3E). A positive correlation was found with CD8 T cell counts in LT for both grA (r = 1.0, $P \leq 0.001$) and perforin (r = 0.87, $P \leq 0.003$) expression in the complete control group (Fig. 4A) A correlation was also evident between the LT incidences of grA-expressing cells and plasma HIV-1 viral load (r = 0.50;

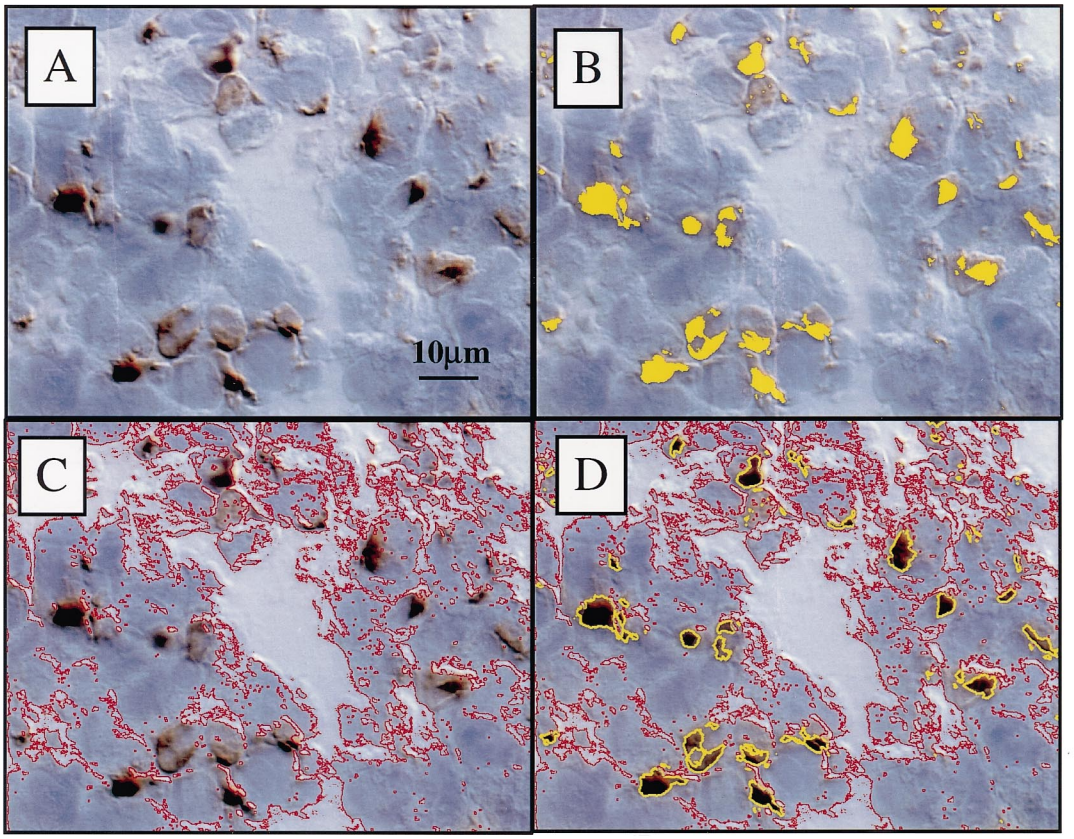

Fig. 1. (A) Digital images illustrating perforin-expressing cells (brown with diaminobenzidine) counterstained with hematoxylin (blue). Lymphocytes expressing perforin in granules are outlined. (B) Individual picture elements (pixel) defining the brown RGB colour thresholds of the peroxidase staining for perforin were calibrated for size in relationship to the magnification (1 pixel = 0.12 μm) and marked in yellow in mask plane 1 by automated computerized image analysis. (C) The total cell area occupied by hematoxylin counterstaining of all cell nuclei was measured in a similar way in mask plane 2 (red contour lines). (D) A superimposed overlay of each of these detected features in mask plane 3 (perforin in yellow, total cells in red) is outlined on the original micrograph. In the presented microscopic field, the perforin-positive stained cell area comprised 6.2% of the total cellular compartment.

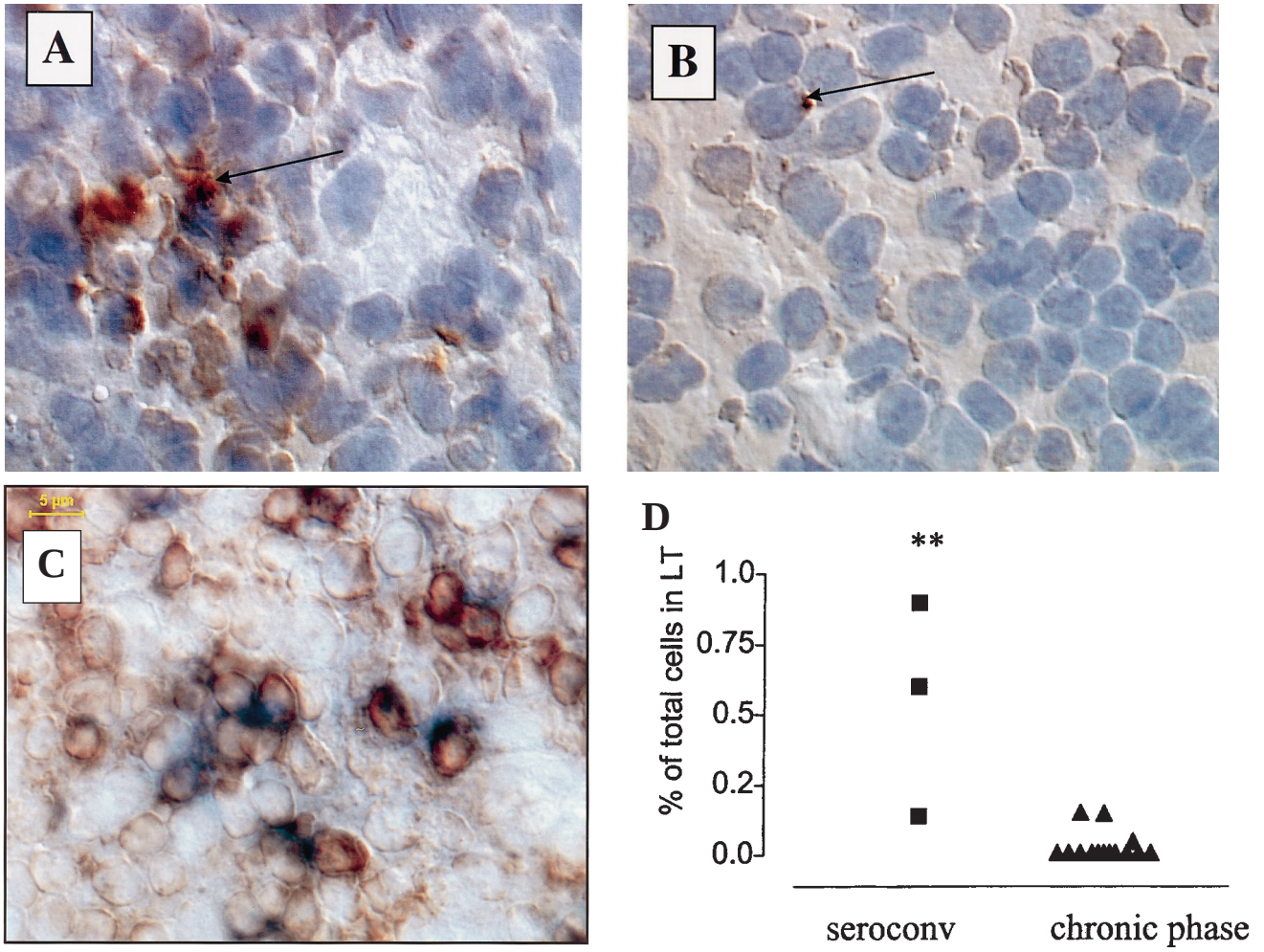

Fig. 2. (A) Photomicrograph showing perforin-expressing cells in lymphoid tissue obtained from a recent HIV seroconverter (4 months from onset of viral syndrome). Perforin (stained brown with diaminobenzidine) had a granular localization. (Original magnification ×400.) (B) A lower incidence of perforin-expressing cells was evident in lymphoid tissue obtained from an asymptomatic patient with long-term infection (stage A1). (Original magnification ×400.) (C) Two-colour staining for phenotyping of granzyme A (grA)-expressing cells within lymphoid tissue obtained from an HIV-infected patient. Over 90% of the grA-expressing cells with a characteristic granule distribution (stained blue by alkaline phosphatase) were CD8 lymphocytes (brown diaminobenzidine cell surface staining). (Original magnification ×400.) (D) Incidences of perforin-expressing cells per total cell area in lymphoid tissue from the HIV-1-infected cohort. A significantly higher proportion of perforin-expressing cells was found in three recent HIV seroconverters compared with 13 patients with established chronic HIV infection (**$P < 0.02$). Quantification of perforin-expressing cells was performed by *in situ* imaging.

$P = 0.05$). In contrast, no association was found in the HIV-infected population between the incidence of perforin expression in LT and CD8 T cells ($r = -0.34$, $P \leq 0.38$) (Fig. 4A). Non-infected control tissue samples had lower CD8 T cell incidences (3–8.6% of total cells) and had very low levels of perforin-expressing cells (0.01–0.1% of total cells) while grA was detected in 0.4-0.9% of all cells (Fig. 3E).

Cytolytic granule expression in blood from HIV-1 patients and controls

PBMC from seven of the HIV-1 infected patients included in the tissue study plus an additional four HIV-positive volunteers were analysed for expression of the two eCTL effector molecules by the *in situ* technique. A significantly higher cellular expression of perforin (13–31% of all PBMC) and grA (14–32% of all PBMC) in PBMC compared with that seen in LT ($P < 0.001$) was noticed in all patients (Fig. 2C). Two-colour staining indicated that > 90% of these perforin- and grA-positive cells were CD8 T cells. The maintenance of perforin expression was not related to HIV disease state. No correlation was found between the percentage of perforin-expressing cells and CD4 cell counts in blood ($r = 0.01$, $P = 0.71$). A relative increase in both grA- and perforin-expressing cells in blood was observed in HIV-positive subjects compared with uninfected controls (11–20%, 12–21% of total PBMC, respectively) (Fig. 4B).

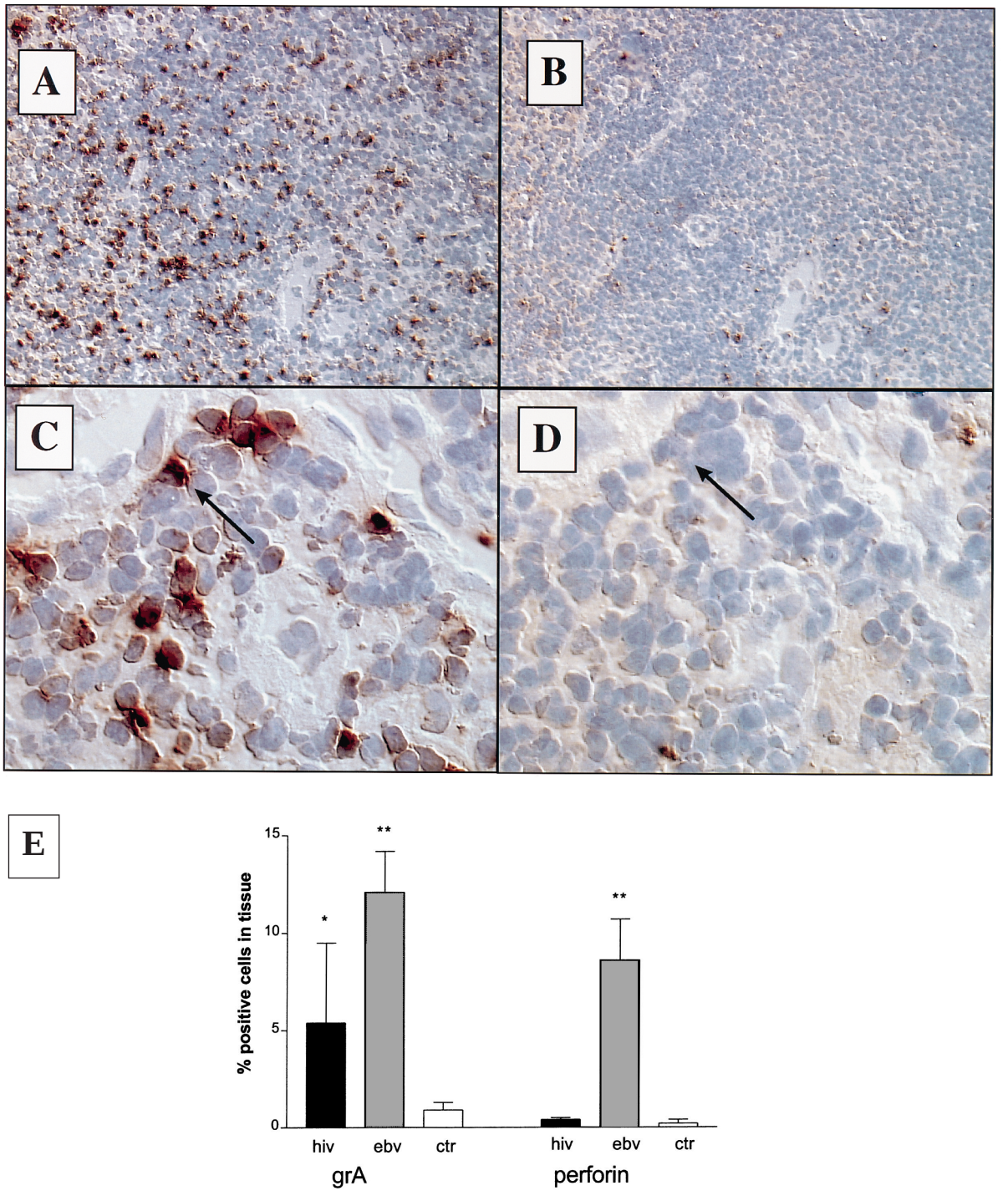

Fig. 3 (A–D) Immunohistochemical staining of a cryopreserved biopsy from a lymph node obtained from a HIV-infected patient with chronic HIV infection (stage A2) showing the dichotomy between the low incidence of perforin-expressing cells (B and D) and higher proportion of granzyme A (grA)-expressing cells (A and C). A and B represent staining from the same tissue area. Perforin- and grA-positive cells were stained brown by diaminobenzidine and the cells were counterstained blue by hematoxylin. Note that grA-expressing cells do not express perforin and vice versa. (Original magnification: A and B × 200; C and D, same area at ×400.) (E) The expression (mean ± SD) of perforin- and grA-expressing cells within lymphoid tissue of HIV-infected patients (n = 16) (hiv), patients with acute infectious mononucleosis (ebv) and controls (n = 4) (ctr) assessed by *in situ* imaging. A significantly higher incidence of grA-expressing cells compared with perforin-expressing cells was evident in the HIV-infected cohort (*$P < 0.05$). Biopsies from patients with acute infectious mononucleosis had a mean incidence of perforin-expressing cells of 8 ± 4% and grA-expressing cells of 12 ± 3%, which was significantly higher than that seen in HIV infection and controls ($P < 0.001$).

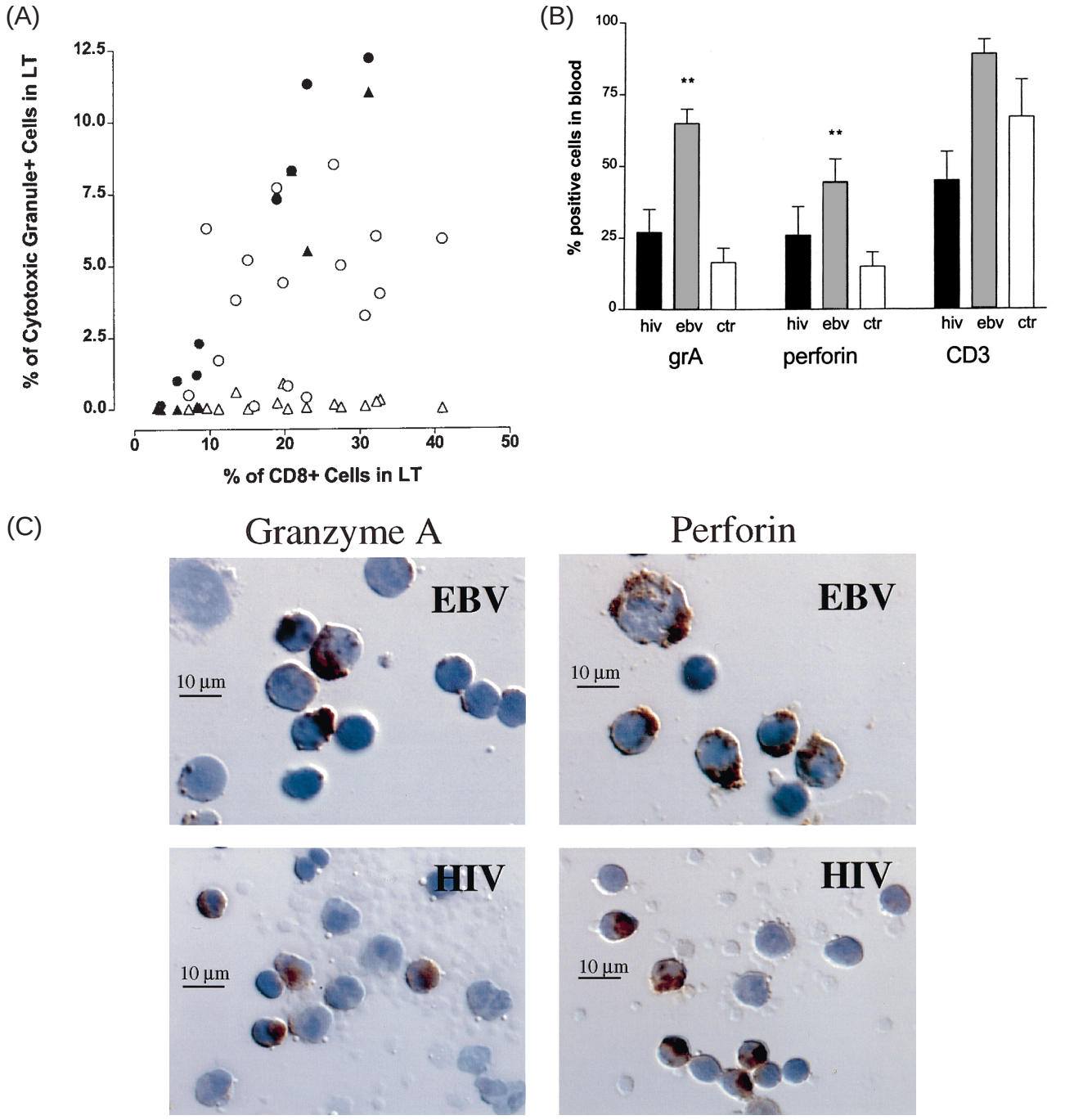

Fig. 4. (A) No correlation was observed with lymphoid tissue CD8 T cell levels for the incidence of perforin-expressing ($r = -0.23$, $P \leqq 0.25$) and granzyme A (grA)-expressing ($r = -0.34$, $P \leqq 0.38$) cells in tissue in 16 HIV-infected patients. In contrast, a strong correlation was found in the control group [infectious mononucleosis (IM), tonsillar hyperplasia (TH)] for both grA ($r = 1.0$, $P \leq 0.001$) and perforin ($r = 0.87$, $P \leq 0.003$) within CD8 T cells in tissue. Statistical evaluation was performed with Pearson correlation. HIV-positive patients: ○, grA; △, perforin. Patients with IM: ●, grA, ▲, perforin; Patients with TH: ●, grA; ▲, perforin. (B) Comparison of cytotoxic granule expression assessed by *in situ* imaging and automated computerized image analysis in fresh PBMC from seven of the sixteen HIV-1 infected study cohort and additionally from our four HIV-infected volunteers and six primary EBV-infected subjects as well as seven controls. Significantly higher incidences of grA and perforin as well as CD3 cells were evident in the cohort with Epstein–Barr virus (ebv) infection ($P < 0.01$). The incidence of grA- and perforin-expressing PBMC obtained from HIV-infected patients (hiv) were comparable and significantly increased compared with healthy controls (ctr) ($P < 0.05$) and were 10- to 100-fold higher compared with levels seen in lymphoid tissue ($P < 0.001$). (C) Blood smears obtained from a patient with primary EBV infection and a HIV-infected patient in chronic phase (stage A2). Cells expressing grA or perforin were stained brown with diaminobenzidine and counterstained blue with haematoxylin.

PBMC from primary EBV-infected patients had a significant increase in both perforin- and grA-expressing T-cells (32–58% and 58–72% of all PBMC, respectively) compared with that seen in HIV-infected patients or in uninfected controls ($P < 0.01$) (Fig. 4B).

Discussion

In the current study, perforin protein expression in LT was more pronounced in patients at early points of HIV seroconversion than in LT from HIV-infected individuals in stages A2 to C. In all stages of HIV infection, the tissue expression of grA-positive cells was significantly higher than that of perforin-positive cells and was not reduced in patients with advanced HIV-1 disease. GrA expression could readily be detected within HIV-infected tissue and differed from perforin levels by at least 10-fold regardless of clinical disease stage (A2–C). The decline of perforin in LT may be a cofactor for the 10- to 100-fold higher viral load detected in LT compared with blood [28]. No association between the level of perforin expression in LT and viral replication could be found in the current study.

In contrast to the deficit in perforin expression seen in tonsil and lymph node biopsies, blood from the HIV-1-infected patients contained abundant numbers of both perforin- and grA-expressing CD8 T cells. This dichotomy between local and systemic perforin synthesis suggests that perforin expression in general was not genetically blocked during HIV infection. The absence of perforin-staining cells at the local site of infection could be caused by eCTL migration out of LT or by CD8 T cell exhaustion, either from recent degranulation or after entering a state of terminal differentiation in which they were unable to produce more effector molecules. Alternatively, HIV proteins, or HIV-infected cells that bind to the CTL receptor but are resistant to lysis, may suppress perforin expression by CTL. It has previously been shown that CTL rapidly downregulate perforin mRNA upon exposure to lysis-resistant, but binding, target cells [29].

Perforin expression occurs in eCTL and natural killer cells in conjunction with grA and grB [10,29,30]. Together, these serine proteases induce apoptosis within virally infected target cells. However, for maximal antiviral activity, perforin must be secreted together with members of the serine granzyme exoprotease family [30]. Perforin is typically observed in blood but can also be induced in all lymphoid sites upon immune activation and acts to form transmembrane pores as channels within the membranes of the target cells [20,29]. The induction of this apopototic process is also dependent upon the presence of perforin for intracellular trafficking of the granzymes [31,32]. This allows the granzyme access to the cytoplasmic target substrates, including caspases, that initiate cell death [31,32]. GrA accumulation in the target cell nuclei and associated DNA fragmentation [24,31] have been shown to be completely blocked in knockout mice lacking perforin, which made these animals highly susceptible to lymphocytic choriomeningitis virus infection [12]. Perforin cannot induce apoptosis by itself [20]. Perforin and granzyme expression occur within days of naive T cell activation [20]. The dominant eCTL mechanism of killing in *in vitro* HIV assays has recently been shown to be granule mediated ([19] and P. Shankar and J. Lieberman, unpublished data). Normally, grA and perforin expression are coordinated and polarized to granules in the eCTL CD8 T cells [30].

The current study suggests that during the course of HIV infection there is a separation of the signals required and delivered for perforin induction from those required for granzyme induction within tissue; alternatively, the cells responding to these signals in tissue have impaired perforin protein expression. This could, in part, be a result of downmodulation of the CD3ζ transducing chain or CD28 cell surface expression. Both these events result in impaired T cell activation and have been documented to occur in CD8 T cells in HIV-infected individuals [7,33]. An additional inhibitor of cytotoxic function produced by CD8+CD57+ lymphocytes has been identified in HIV-infected and allogeneic bone marrow transplant recipients [34].

Furthermore, it has previously been reported that HIV-1-infected patients express elevated levels of interferons α and γ and the interleukins 1, 12 (p40 and p70 heterodimers) and within lymphoid sites [23]. It is difficult to explain why impaired perforin production occurs in an environment of upregulated type 1 cytokines (IL-2, IFNγ)and β-chemokine production (Rantes, Mip 1α, Mip 1β). Reduced expression of CD3ζ, CD28 andCD40 ligand found in chronic HIV-1 infection [35,36] may, therefore, be a consequence of HIV-1 genes triggering abnormal immune activation, antigenic persistence, loss of cognate CD4 help or the chronic proinflammatory state. The end result is unsynchronized perforin versus granzyme expression in the LT, which affects the perforin/granzyme eCTL pathway in one of the primary sites of HIV-1 replication.

Acknowledgements

The authors wish to thank Drs Alden Harken and John Pottage for performing lymph node biopsies.

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