

Oligoclonal Expansion of HIV-specific Cytotoxic CD8 T Lymphocytes in the Skin of HIV-1-infected Patients with Cutaneous Pseudolymphoma

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Abstract

A massive infiltration of the skin by activated CD8⁺ T lymphocytes involving both the dermis and the epidermis has been found in HIV-1-infected patients presenting with a chronic skin rash. We characterized the T cell receptor (TCR) BV-BJ junctional diversity of the skin-infiltrating lymphocytes (SILs) in four patients. The SILs expressed a limited set of TCRBV gene segments. Complementarity determining region 3 length analysis further emphasized their oligoclonality, suggesting that antigen stimulation might be responsible for the cutaneous T cell expansion. Furthermore, independent skin biopsies obtained from the same individual were shown to harbor distinct T cell repertoires, possibly reflecting the spatial heterogeneity of the antigenic stimuli. The CD8⁺ cytotoxic T lymphocyte (CTL) lines isolated from the skin rash in one patient exhibited a specific, class I MHC-restricted cytotoxic activity against HIV-1 Gag- and Pol-expressing target cells, whereas CTL lines derived from the skin lesions of a second patient were shown to be predominantly Env-specific. Taken together, these data demonstrate the infiltration of HIV-specific CTLs in the skin of HIV-infected patients, and suggest that in addition to their known role in controlling the retroviral infection, these CTLs may also be involved in the pathogenesis of cutaneous inflammatory disorders occurring during the course of HIV infection. (*J. Clin. Invest.* 1998. 101:2506–2516.) Key words: HIV-1 • cytotoxic T lymphocyte • T cell receptor BV • repertoire • rash

Introduction

CD8⁺ cytotoxic T lymphocytes (CTLs)¹ play a key role in the defense against intracellular pathogens (1, 2). In mice, virus-specific CTLs have been shown to be involved in the eradication of respiratory syncytial virus (3), whereas in humans, virus-specific CTL activity protects against influenza (4). After

primary HIV infection, oligoclonal expansion of HIV-specific CTLs can be detected in the peripheral blood (5). Moreover, extensive studies have documented a CTL response against viral peptides derived from structural or regulatory HIV proteins, in the blood (6–8) and in the lymphoid organs (9–11) of HIV-infected patients. Although specific cytotoxic cells fail to eliminate all HIV-infected cells, they are widely believed to be essential to the control of HIV replication during the asymptomatic phase of HIV infection, by killing infected cells before virion release (12).

The antiviral immune response can also cause local tissue damage, as has been demonstrated for the brain lesions of mice infected with the lymphocytic choriomeningitis virus (13), and for the liver necrosis of hepatitis B virus-infected humans (14). In a similar way, the contribution of a specific CTL response to other sequelae during viral infection has been suggested in patients affected by HIV-related lymphocytic alveolitis (6), and in simian immunodeficiency virus (SIV)-infected rhesus monkeys presenting with skin eruption (15).

Recently, a new disease entity consisting of a chronic severe skin disorder characterized by extensive plaque lesions or erythroderma has been reported in patients with AIDS (16, 17). In patients presenting with this syndrome, histopathological examination of skin biopsies revealed a dense T cell infiltration of the dermis, with or without epidermal involvement (18). Although these morphological findings may be suggestive of a cutaneous T cell lymphoma (CTCL), i.e., mycosis fungoides or Sézary syndrome, results from immunophenotypic studies of the skin infiltrate and from clonality assays have shown major differences from CTCLs occurring in HIV-seronegative patients (16–18). First, the tumoral cells in “classical” CTCL usually exhibit a CD4 T helper phenotype (19), whereas dermal and epidermal lymphocytes in HIV-associated cutaneous pseudolymphoma express predominantly the CD8 antigen (16–18). Furthermore, whereas CTCLs result from the monoclonal proliferation of malignant lymphocytes (20, 21), molecular genetic studies in AIDS-associated CTCL-like disease failed to detect any dominant monoclonal subset (16, 19), thus questioning the neoplastic nature of these lymphoproliferations.

In this study, we analyzed the repertoire of BV segments used by skin-infiltrating CD8 cells in four HIV-infected pa-

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1. *Abbreviations used in this paper:* CDC, Center for Disease Control; CDR3, complementarity-determining region 3; CLA, cutaneous lymphocyte-associated antigen; CTL, cytotoxic T lymphocyte; CTCL, cutaneous T cell lymphoma; LC, Langerhans cell; PE, phycoerythrin; RT, reverse transcriptase; SIL, skin-infiltrating lymphocyte; SIV, simian immunodeficiency virus; TCR, T cell receptor; VAC-ENV, -GAG, -NEF, and -POL, recombinant vaccinia virus expressing HIV-1 LAI gp160, HIV-1 LAI Gag, HIV-1 LAI Nef, and HIV-1 LAI Pol; VAC-WT, wild-type vaccinia virus, Copenhagen strain.

Table I. Clinical Profile, and Immunological and Virological Status in the Four HIV-infected Patients

Patient	Age/sex	CDC stage	PBMC counts		p24 antigenemia	Viral load*
			CD4 cells/ μ l	CD8 cells/ μ l		
A	32/M	A	450	1800	Negative	1500
B	43/M	B	5	523	Negative	23500
C	62/M	C	14	464	Positive (225 pg/ml)	ND
D	50/M	C	4	221	ND	9700

*Measured in the patients' serum using HIV RNA-specific RT-PCR (Amplicor test). ND, Not determined.

tients affected by this syndrome. Our analysis of the T cell receptor (TCR) β junctional sequences strongly suggest a complementarity-determining region 3 (CDR3)-dependent cutaneous expansion of particular BV segments. We further examined the functional profiles of CD8⁺ cell lines derived from the skin of two patients. These studies showed that these lines were involved in a specific, HLA class I-restricted cytotoxic recognition of HIV antigens.

Methods

Patients. The study was performed after obtaining the informed consent of four HIV-1-infected male patients presenting with a chronic erythrodermic cutaneous disorder. The immunological status and virological parameters obtained in these patients at the time of diagnosis of skin disease are listed in Table I. Patients did not receive any therapy at the time of onset of skin lesions, which consisted initially of erythroscumous plaques, progressively resulting in erythroderma. Pigmented lesions developed in patients A, B, and C during the course of the disease (Fig. 1). Skin symptoms followed a chronic course, their duration lasting from < 6 mo to 2 yr before this study.

All patients received topical corticosteroids without efficacy before the study, and patient A also received Psoralen photochemotherapy (PUVA therapy), which led to a worsening of skin symptoms. Local corticosteroid therapy was stopped at least 15 d before skin biopsies. Histopathological analysis of skin biopsies revealed in all cases a dense dermal infiltration by T cells, together with epidermal exocytosis. In some biopsies, cytonuclear irregularities were observed throughout the skin infiltrate, supporting the diagnosis of CTCL-like infiltration of the skin (18). In all biopsies, dermal and epidermal infiltrating lymphocytes exhibited exclusively a mature CD8⁺ CD4⁻ phenotype. In patient A, histopathological examination of a biopsy taken from an enlarged cervical lymph node, performed 3 mo after the diagnosis of skin disease, showed an interfollicular hyperplasia, without evidence of lymphoma. Patient A was classified in group A according to the Center for Disease Control (CDC) criteria (22); patient B was CDC group B (oral hairy leucoplakia and oral candidiasis); and patients C and D were CDC group C (*Pneumocystis carinii* pneumonia/*Toxoplasma gondii* infection and *Mycobacterium tuberculosis* infection, respectively) (Table I). Control patients E and F were two HIV-1-infected asymptomatic patients showing CD4 cell counts of 423 and 254 cells/ml and CD8 cell counts of 678 and 602 cells/ml, respectively.

Immunohistochemistry. Immunohistochemical staining of skin punch biopsies and of the lymph node biopsy was performed on frozen sections using a standard avidin-biotin immunoperoxidase procedure described previously (23), with the mAbs anti-CD3, -CD4, -CD8, -CD2, -CD5, -CD7, -HLA DR, -CD38, -CD16, -CD56, -CD57 (Immunotech, Marseille, France); 1C1 (anti-BV5.2/5.3), W112 (anti-BV5.3), OT145 (anti-BV6.7), 16G8 (anti-BV8), SS11 (anti-BV12), LC4 (anti-BV5.1) (all anti-TCR β chains from T Cell Sciences, Inc., Cambridge, MA); HECA-452 (anti-cutaneous lymphocyte-associated antigen [CLA]; PharMingen, San Diego, CA); and Kal-1 (anti-HIV-1 capsid protein p24; DAKO Corp., Carpinteria, CA).

Flow cytometry. For double-fluorescence analysis of T cell lines and PBMCs, cells were stained with the following mAbs: phycoerythrin (PE)-conjugated anti-CD8, PE-conjugated anti-CD4 (Immunotech), FITC-conjugated anti-CD4, and FITC-conjugated anti-CLA (PharMingen). For each sample, 10⁵ cells were incubated for 20 min at 4°C, washed with PBS, fixed with 2% paraformaldehyde in PBS, and analyzed on a FACScan[®] flow cytometer (Becton Dickinson, San



Figure 1. Infiltrated erythroderma with pigmented lesions on the dorsal area of patient A.

Jose, CA). 10,000 events per sample were collected and analyzed using Cellquest software (Becton Dickinson).

Analysis of the repertoire of TCRBV genes. The TCRBV repertoire was analyzed by a semiquantitative PCR assay as described previously (24). Briefly, total RNA was extracted from cells (skin biopsies, purified CD8⁺ PBMCs, CD8⁺ cell lines) by the method of Chomczynski and Sacchi (25). 8 µg of RNA was reverse transcribed in a 50-µl reaction volume using the reagents provided in the Stratascript reverse transcription (RT) PCR kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. A Cβ1 and Cβ2-specific 3' oligonucleotide, 5'-CGGGCTGCTCCTTGAGGGGCTGCG-3', and 1 of 24 different BV family-specific oligonucleotides (26) were used, together with a primer pair specific for the TCR Cα chain (27) as an internal PCR control. 2 µl of the RT product was incubated in a final reaction volume of 50 µl containing 10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl, pH 8.3, 20 pmol of each oligonucleotide, 0.2 mM of each dNTP, and 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim, Mannheim, Germany) which had been blocked for "hot start" with 1.1 µg of TaqStart antibody (Clontech, Palo Alto, CA). After an initial denaturation step of 3 min at 95°C, the reactions were subjected to 30 cycles of PCR (94°C for 30 s, 60°C for 1 min, 74°C for 1 min), followed by a final extension step of 5 min at 74°C. PCR products were analyzed on 2% agarose gels containing 0.5 µg/ml ethidium bromide. The bands were visualized on an ultraviolet (UV) light box and photographed using a computerized image capture system (Image Store 5000; UVP Ltd., Cambridge, UK). Specificity of amplification was assessed by Southern blot, after transfer on nylon membranes and probing with a ³²P-end-labeled Cβ-specific internal oligonucleotide (27). Specific signals were quantified by scanning membranes using a Parallel Plate Avalanche Charnpak chamber (β Imager 1200; Biospace Instruments, Thoiry, France) and β-Vision software (Biospace Instruments). Results are expressed for each BV family as a percentage of the sum of all 24 BV family signals measured.

Run-off reactions. One nested Cβ primer, 5'-GTGCACCTCTCTCCATTCA-3', was dye-labeled (Joe Fluorophore; Applied Biosystems, Inc., Foster City, CA) and used in run-off reactions. 2 µl of the PCR product was added to 8 µl of a mixture containing 10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl, pH 8.3, 0.2 mM of each dNTP, 0.5 U of *Taq* DNA polymerase, and 0.1 µM of the Joe-labeled Cβ oligonucleotide. The extension reaction consisted of a 3-min denaturation step at 95°C followed by 12 cycles of 30 s at 94°C, 30 s at 60°C, and 2 min at 72°C. A final 10-min incubation at 72°C was performed.

Electrophoresis and quantitative analysis of TCR CDR3 lengths. 10 µl of a 5:1 mixture of formamide and 50 mg/ml dextran blue/25 mM EDTA (pH 8.0) were added to the 10 µl run-off product. 2 µl of this mixture was loaded on a 4% acrylamide/4 M urea sequencing gel and run on a DNA sequencer (ABI 377; Applied Biosystems, Inc.). A mixture of dye-labeled size standards was also loaded on the sequencing gel to allow precise determination of the sizes of the BC-BV or BC-BJ run-off reaction products. The size and area of the peaks corresponding to the DNA products were determined using the Immunoscope software package (version 3.01c; C. Pannetier, Pasteur Institute, Paris, France). The observed peaks are usually separated by three bases and correspond to in-frame transcripts of TCRs. Windows of analysis were centered on expected sizes corresponding to TCR transcripts encoding a 10-amino acid-long CDR3. The CDR3 region was defined according to Kabat et al. (28).

Cloning and sequencing of PCR products. BV amplicons of interest were gel purified and ligated directly into the PCR[®]II vector (TA cloning kit; Invitrogen Corp., San Diego, CA). After amplification into *Escherichia coli*, plasmids were purified, and their inserts were sequenced using an automated sequencer with M13 rev and T7 dye-labeled primers and *Amplitaq* DNA polymerase FS (Applied Biosystems, Inc.).

Target cells. An EBV-transformed B lymphoblastoid cell line (B-LCL) was established from PBMCs isolated from patient A and expanded in RPMI 1640 supplemented with 10% FCS, L-glutamine,

penicillin 100 U/ml, and streptomycin 100 µg/ml. B-LCL cells derived from healthy donors and selected for HLA antigens matched with the patients were also used as target cells. B-LCL cells were infected with recombinant vaccinia virus constructs expressing different HIV gene products, at an moi of 5 PFU per cell. Recombinant vaccinia virus constructs included the wild-type Copenhagen strain (VAC-WT) and various recombinants encoding either the Gag (VAC-GAG), Env (VAC-ENV), Pol (VAC-POL), or Nef (VAC-NEF) antigens of the HIV-1 LAI isolate (Transgène, Strasbourg, France) (29, 30).

Effector cells. Skin lymphocytes were isolated from two patients as described previously (31). Briefly, a 5-mm punch skin biopsy was rinsed and minced gently with scissors in RPMI 1640 medium (ICN Biomedicals, Inc., Costa Mesa, CA), then filtered through a sterile gauze. The PBMCs were isolated using Ficoll-Hypaque density gradient centrifugation (EuroBio S.A., Les Ulis, France). CD8⁺ cell lines were obtained by immunomagnetic purification of PBMCs with anti-CD8-coated immunobeads (Dynal A.S., Oslo, Norway). Effector cells were incubated in round-bottomed 96-well plates (Costar Corp., Cambridge, MA) with 5×10^5 /ml 5,000 rads γ -irradiated autologous PBMCs previously stimulated overnight with 1 µg/ml phytohemagglutinin (PHA; Murex Diagnostics, Dartford, UK), in RPMI medium supplemented with 10% human serum AB (SAB), 0.3 mg/ml glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin (GIBCO BRL, Cergy-Pontoise, France). Cell lines were maintained at 37°C in a humidified atmosphere of 5% CO₂. Recombinant human IL-2 (20 IU/ml; Boehringer Mannheim) was added at days 3, 9, and 15, and CTL responses were tested at day 21.

Human HLA typing. HLA class I and II transplantation antigens were defined serologically using a standard complement-mediated microcytotoxicity assay. The typing of class II antigens was further confirmed using RFLP procedures.

Cytotoxicity assays. Target cells were labeled with Na₂⁵¹CrO₄ (Amersham International, Les Ulis, France) at 80 µCi/10⁶ cells. Suspensions of 4×10^5 target cells were added in triplicate to microtiter plates before addition of effector cells at various E/T ratios. After a 4-h incubation period at 37°C, the supernatants were harvested, and the chromium release was measured in a γ counter (Clinigamma; ECG Instruments, Evry, France). Spontaneous ⁵¹Cr release values were 15–25% of the total incorporated radioactivity. The percentage of specific ⁵¹Cr release was calculated as follows: $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{total release} - \text{spontaneous release})$.

Results

Phenotype of skin-infiltrating cells in HIV-infected patients presenting with the cutaneous pseudolymphoma. Using immunohistological analysis, cells infiltrating the dermis and/or epidermis of the four patients were shown to be comprised of > 90% CD2⁺ CD3⁺ TCRαβ⁺ cells (Fig. 2), expressing exclusively CD8 (17). In patient A, ~ 30% of these CD8⁺ cells were stained by the mAb S6F1, which reacts with the cytotoxic T cell subset (6). In all cases, the proportion of CD25⁺ cells among skin-infiltrating lymphocytes (SILs) was < 5% (data not shown). Immunostaining analysis with a panel of anti-BV mAbs in patient A showed that ~ 20% of SILs were stained with an anti-BV8 (Fig. 2), whereas only 4% of CD8⁺ PBMCs were shown to be BV8⁺ by double-staining immunofluorescence analysis (Table II). Immunostaining of the lymph node biopsy taken from patient A showed a T cell infiltration of the interfollicular areas composed of CD4⁺ and CD8⁺ cells (Fig. 3, A and B). Interestingly, BV8⁺ lymphocytes were also observed, representing ~ 10% of the lymph node T cell infiltrate, and HIV-1 p24⁺ cells were detected, some of them localized in the vicinity of CD8⁺ cells (Fig. 3, C and D). Immuno-

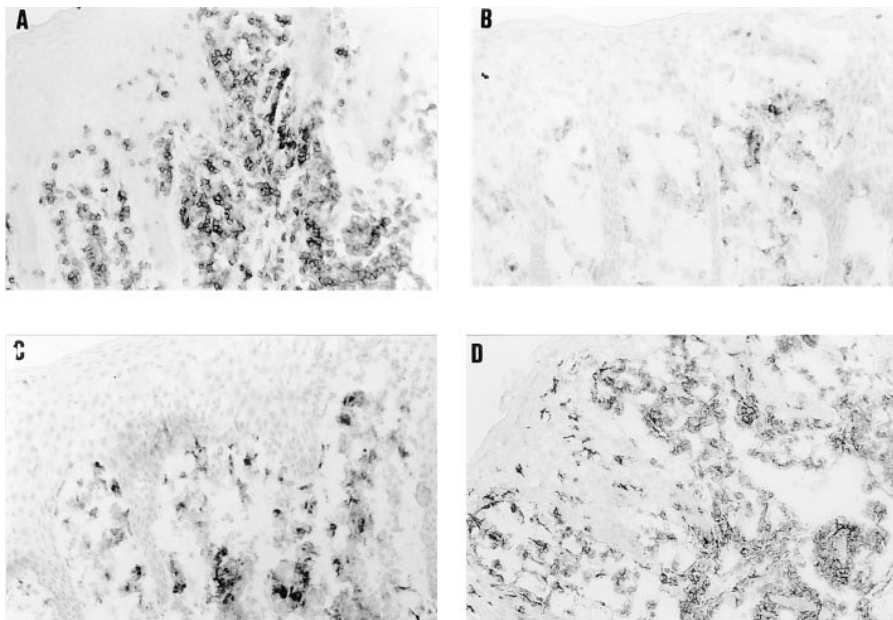


Figure 2. Frozen tissue sections from skin lesions of patient A, stained with either anti-CD3 mAb (A), anti-BV8 mAb (B), anti-S6F1 mAb (C), or anti-CLA mAb (D) and counterstained with hematoxylin. Most of the dermal and epidermal infiltrating T cells are CD3+ CLA+.

staining with HECA-452 mAb, which reacts with the skin-homing receptor CLA (32), was performed on the lesional skin and on PBMCs from patient A. The results showed that all SILs expressed CLA (Fig. 2 D), whereas in the blood, CLA expression was predominant in the CD8+ cell subset (Table II). Furthermore, cytofluorometric analysis of PBMC subsets from four HIV-infected patients without skin symptoms showed that the mean proportion of blood lymphocytes coexpressing CLA and CD8 was far below that found in patient A (1.8 vs. 16%, respectively) (Table II).

Skin-infiltrating CD8+ T cells use a restricted set of TCRBV gene segments. To determine whether an antigenic stimulation could be involved in the recruitment, activation, and expansion of CD8+ cells in the skin rash occurring during the course of

HIV infection, we looked for a preferential use of particular TCRBV segments by the SILs in four patients affected by this cutaneous disorder. We compared the TCRBV repertoire used in SILs and in CD8+ PBMCs by semiquantitative PCR analysis of TCR β transcripts belonging to the 24 TCRBV families. As shown in Table III, comparison of the TCRBV repertoires from the skin and blood of each patient established that particular BV segments were used preferentially by infiltrating cutaneous lymphocytes. In patient A, BV2 and BV16 transcripts are twice as abundant in the skin, compared with blood; BV6 and BV8 transcripts were also overrepresented in the skin. Similarly, a bias towards BV16 expression was found in SILs from patient B, whereas transcripts belonging to that family were not predominantly amplified from normal skin

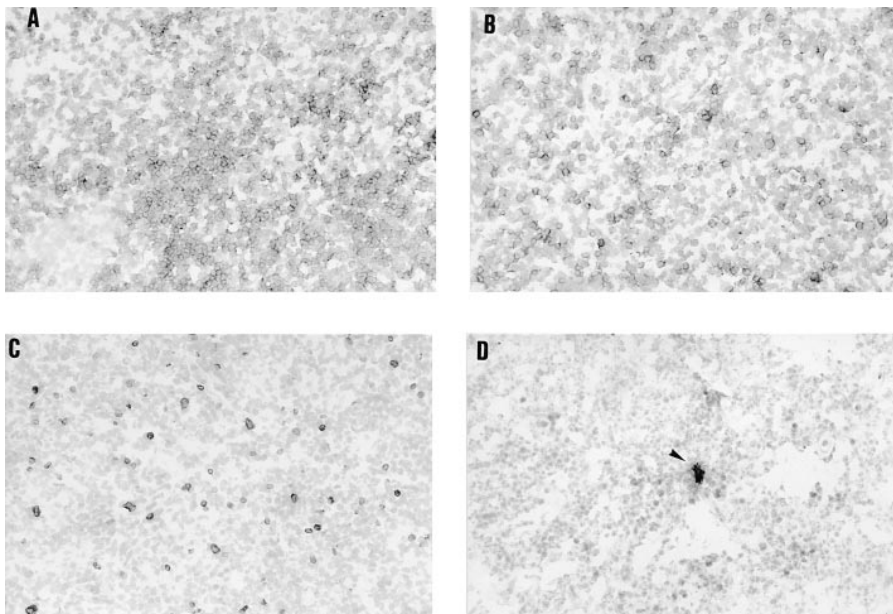


Figure 3. Frozen tissue sections from the cervical lymph node biopsy performed in patient A. Immunostaining with anti-CD8 mAb (A), anti-CD4 mAb (B), anti-BV8 (C), or anti-HIV p24 (D). p24-stained cells (arrow) are present in the interfollicular area, which is infiltrated by T lymphocytes that are predominantly CD8+.

Table II. Immunostaining Analysis of CLA Expression Among CD4+ and CD8+ PBMCs of Patient A, and of Four HIV-infected Patients without Skin Symptoms

Patient	PBMC counts		Percentage of blood lymphocytes expressing*			
	CD4	CD8	CD4+	CD8+	CD4+ CLA+	CD8+ CLA+
	cells/ μ l	cells/ μ l				
A	450	1800	17	56	1.7	16
G [‡]	123	560	11.8	60.3	0.97	0.59
H [‡]	139	529	17.5	63.55	0.64	0.77
I [‡]	185	554	18.8	57	1.51	1.77
J [‡]	312	540	25.1	54.2	0.9	3.10

*Results are obtained by flow cytometry analysis of PBMCs after double immunostaining with a PE-conjugated anti-CD8 or anti-CD4 mAb and with an FITC-conjugated anti-CLA mAb; expressed as a percentage of all gated lymphocytes. [‡]Patients G, H, I, and J are four HIV-seropositive individuals devoid of any skin disease at the time of the study.

Table III. Percentage of BV Gene Expression in SILs and CD8+ PBMCs from HIV-infected Patients with a Chronic Cutaneous Disorder

BV family	Patients									
	A			B		C			D	
	SIL1	SIL2	PBMC	SIL	PBMC	SIL1	SIL2	PBMC	SIL	PBMC
1	11	5.9	10	5.8	11.9	5.4	6.8	3.7	3.3	5.6
2	13.8*	14.7*	6.6	10.5	15	5.4	7.8	5.1	18.3*	7
3	2.1	4.4	8.2	5.8	10.1	1.8	6.8	6.9	4.2	5.3
4	12.2	4.4	9.1	12.7	11	4.8	29.4*	5.5	18.3*	7.3
5	7.8	4.9	2.2	3.4	11.6	9	5.9	5.5	16.6*	4.8
6	10.2	12.2	11.9	0	5.8	9.6	6.8	18.4	8.3	8.4
7	6	3.9	7.2	4.6	4.3	7.2	7.8	13.8	12.5	7
8	14.8*	2.9	5.4	5.8	4.9	41*	19.6*	4.2	5	8.2
9	2.4	4.9	3.4	0	2.3	0	0	0	0	4.2
10	0.4	1.5	1.8	0	0	1.2	0	0	0	2.2
11	0	1.5	3.6	0	0.9	1.8	0	4.6	0	3.7
12	2.9	9.3	11.6	0	1.4	0	0	0	0	1.7
13	0.2	2.4	1	0	0.4	1.8	0	0	0	1.4
14	1	6.4*	1.3	0	1.7	2.4	0	0	1.7	0.6
15	0.8	2.9	3.2	4.6	2.3	3	0	3.2	2.5	4.5
16	8.6*	3.4	2.6	40.7*	3.8	1.8	0	3.2	1.7	4.2
17	2.9	2.9	2.3	5.8	4.9	1.8	2.9	3.7	0	3.4
18	0	0	0	0	0	0	0	0	0	0.6
19	1.8	3.4	2.5	0	0	0	1.9	6	4.2	3.7
20	0	0	0	0	1.6	0	0	2.4	2.5	1.4
21	0	0	0	0	0	0	0	7.8	0	0
22	0	2.4	0	0	0.4	0	0	4.2	1.7	5.3
23	0.1	5.4	1.9	0	3.5	1.8	3.9	8.4	2.5	5.1
24	0.5	0	0	0	2	0	0	0	0	4.2

Results are expressed as the percentage of total BV PCR products, and are compared within the same patient between skin lymphocytes and CD8+ PBMCs. In patients A and C, two separate skin samples (SIL1 and SIL2) were analyzed. *Skin values at least twice as large as the corresponding ones in paired PBMCs.

samples obtained in two HIV-seropositive asymptomatic individuals (Table IV). Among SILs from patient C, expression was skewed towards the BV8 family. In patients A and C, TCRBV transcripts were analyzed in skin samples taken from anatomically distinct sites. Interestingly, striking changes in the relative expression of transcripts specific for particular BV families were evident from one area of affected skin to another in the same patient, as shown for the BV4 and BV8 subsets in patient A, and for the BV4 subfamily in patient C (Table III).

Altogether, this first set of data strongly suggests a skin-specific homing and expansion of CD8+ T cells expressing particular TCRBV gene segments in HIV-infected patients with a lymphoinfiltrative disorder of the skin.

Oligoclonal nature of CD8+ SILs. BV-specific PCR only allows a low-resolution analysis of the TCR repertoire. Indeed, the fine antigenic specificity of the TCR is carried almost entirely by the CDR3 (33), which can vary by as many as eight residues between different TCR β chains.

To address whether the CD8+ SILs in these HIV-infected patients could be involved in an antigen-specific immune response, we comparatively studied the TCRBV junctional diversity used by the SILs and by the PBMCs in the BV families that were found to be overrepresented in the SILs. It was observed with the so called “immunoscope” approach that BV-BC PCR products yield an average of eight peaks spaced

Table IV. Percentage of BV Gene Expression in Lymphocytes Infiltrating the Normal Skin (SIL) from Two Asymptomatic HIV-infected Control Patients

BV family	Controls	
	E	F
1	10.2	8.4
2	13	21.8
3	7.6	23.1
4	9.25	14.7
5	7.1	7.5
6	5.9	0
7	8.8	4.2
8	9.5	4.6
9	2.4	1.2
10	0.5	2.5
11	0.7	2.1
12	0.6	0
13	0	0
14	0.3	0.2
15	6.6	7.1
16	4.5	0.4
17	5	0
18	0	1.2
19	1.9	0
20	0	0
21	0.9	0
22	1.4	0.8
23	0.2	0
24	1.1	0

Results are expressed as the percentage of total BV-BC PCR signals.

by three nucleotides (corresponding to in-frame transcripts). Moreover, it was also shown that in normal PBMC samples, BV-BC size distribution profiles usually have Gaussian patterns that signify polyclonality, and are remarkably stable between control subjects over time (34). Conversely, a relative increase in the height of a particular peak that disrupts the Gaussian pattern by surfacing over polyclonal background usually corresponds to a clonal expansion, as has been demonstrated by direct sequence analysis of such "abnormal" peaks (34).

CDR3 size distribution analysis revealed the presence of few dominant peaks among patterns obtained from SILs, whereas the corresponding CD8+ PBMC profiles were more diverse (Fig. 4). Immunoscope analysis of SIL samples from patient A revealed the presence of two strikingly dominant peaks corresponding to TCR β chains, characterized by their CDR3 lengths of eight and nine amino acids within the BV6 and BV16 subsets, respectively. As indicated in Fig. 4, dominant peak sizes were demonstrated within SIL TCR BV4 and TCR BV16-specific products from the skin of patient B, TCR BV4 and TCR BV8 from the skin of patient C, and TCR BV4 from the skin of patient D. Altogether, the TCR β CDR3 size distribution patterns strongly suggest the oligoclonal nature of CD8+ T cells infiltrating the skin lesions. In patients A and C, skin biopsies were performed simultaneously at two separate sites of involved skin. Immunoscope analysis of TCR transcripts amplified from these samples revealed clear topographic differences in TCRBV repertoire used by SILs within the same individual. As shown in Fig. 4, SILs from patient A make use of totally dissimilar repertoires at distinct skin areas.

Although clear differences are also found in BV8 immunoscope profiles obtained from the two biopsies of patient B, a similarly biased repertoire of BV4 gene segments was used by SILs from the same subject, in both areas.

Analysis of the TCRBV CDR3 sequences in the HIV-related skin disorder support an antigen-driven selective expansion of SILs. We cloned and sequenced the BV-BC PCR products obtained from blood and skin lesions of patient A, with oligonucleotides specific for TCRBV families BV2, BV6, BV8, and BV16, i.e., families found to be overexpressed in SILs. As shown in Table V, there was a clearly biased use of the BJ2S1 segment within BV2 SILs in patient A, since eight of nine in-frame BV2 rearrangements used the BJ2S1 segment, and three isolates showed an identical CDR3 amino acid sequence. Five additional clones exhibited a conserved glutamic acid-glutamine motif (EQ) contributed by the BJ segment. In contrast, the five BV2 clones from the CD8+ PBMCs showed no redundant CDR3 DNA or amino acid sequence. Interestingly, all the sequences of BV2 CDR3 regions cloned from the blood were distinct from those derived from the SILs. In the same patient, the BJ2S7 segment was used by seven of eight BV6 clones derived from the SILs, six of them bearing an identical CDR3 including the EQ motif from the BJ segment. As expected from immunoscope analysis, the CDR3 of the dominant BV6 clone was six amino acids long (see Fig. 4, patient A, sample 1). However, all BV6 cDNA clones derived from the CD8+ PBMCs were different, and displayed CDR3 sequences that were not found in the skin sample. Two clones appeared to dominate the BV8+ subset of the skin infiltrate. Finally, all BV16 sequences derived from SILs were identical, as expected

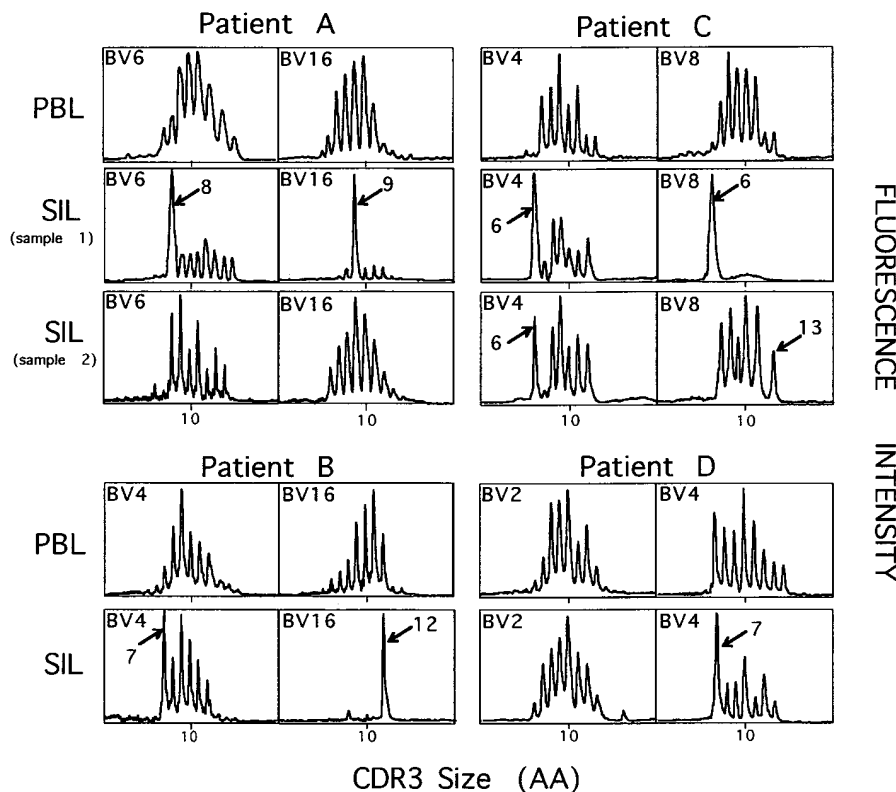


Figure 4. The SIL population is likely to be strongly oligoclonal and compartmentalized. Immunoscope profiles corresponding to BV families overexpressed in the skin (see Table III) are represented in comparison with those obtained from autologous CD8+ PBMCs. For each patient, two BV families were studied, and the corresponding BV-specific primer used is indicated in the window of analysis. The CDR3 length of the major size peaks detected is indicated (arrow, and number of amino acid residues). Two separate skin samples (right and left abdominal flanks, sample 1 and sample 2, respectively) were studied in two cases. The graphs represent the intensity of fluorescence in arbitrary units, as a function of the CDR3 length, in amino acids (AA), of BV-BC single-strand DNA run-off products.

Table V. DNA Sequences of BV-BJ TCR Junctional Regions Cloned from PCR Products Obtained from Skin Samples (SIL) and from Purified CD8+ PBMCs of Patient A, and Predicted Amino Acid Sequences of the CDR3

Source	BV	BJ	Vβ	CDR3 region	Jβ	CDR3 AA sequence	Frequency
SIL	2	2S1	TGCAGTGTCT	AGAGATCACAGCGGGGCTCTGGAATGAGCAG	TTCTTCGGG	RDHSGASGNEQ	3/9
	2	2S1	TGCAGTGTCT	CTGATCATAAGCTCCTACAATGAGCAG	TTCTTCGGG	LIISYNEQ	1/9
	2	2S1	TGCAGTGTCT	ATACCGGGACAAATCTACAATGAGCAG	TTCTTCGGG	IPGQIYNEQ	1/9
	2	2S1	TGCAGTGTCT	ACTCGTGAGACTAGCGGGGGGGGAGCAG	TTCTTCGGG	TRETSGGGEQ	1/9
	2	2S2	TGCAGTGTCT	CTCACCGGGGACAGGGAGCTGTACGGGGAGCTG	TTTTTTGGA	LTGDRELYGEL	1/9
	2	2S1	TGCAGTGTCT	AGAGAACCAACTAGCGGGAGGGCTATGGATGAGCAG	TTCTTCGGG	REPTSGRAMDEQ	1/9
	2	2S1	TGCAGTGTCT	AGGGACTGGCGGGAGCGGAGCTCTACAAGAGCAG	TTCTTCGGG	RDWRERSYKEQ	1/9
CD8 PBMC	2	2S3	TGCAGTGTCT	CGGCTCCGGGCCCCAAGCACAGATACGCAG	TATTTTGGC	RLRAQSTDTQ	1/5
	2	2S1	TGCAGTGTCT	AACCTCCCCCTGAGCAG	TTCTTCGGG	NLPPEQ	1/5
	2	2S1	TGCAGTGTCT	ACTCGTGAGACTAGCGGGGGGGAATGAGCAG	TTCTTCGGG	TRETSGGGNEQ	1/5
	2	1S3	TGCAGTGTCT	ACCGGGGACAGATTTGGAAACACC	TATTTTGGG	TRDRFGNT	1/5
	2	2S1	TGCAGTGTCT	AGAGATAAGGCTAGCGAGCTATACAATGAGCAG	TTCTTCGGG	REKASELYNEQ	1/5
SIL	6	2S7	TGTGCCAGC	AGCTTAGGACAAGCCTACGAGCAG	TACTTCGGG	SLGQAYEQ	6/8
	6	2S3	TGTGCCAGC	AGCTCGCAACTGACCGGGACTAGAGGGACAGATACGCAG	TATTTTGGC	SSQLTGRGTDQ	1/8
	6	2S7	TGTGCCAGC	AGCGTTTCTGAACCGGAGCAG	TACTTCGGG	SVSEPEQ	1/8
CD8 PBMC	6	2S1	TGTGCCAGC	AGCCAAGCGGCGCTCGGGACAGGGCTTTACAATGAGCAG	TTCTTCGGG	SQAAVGTGLYNEQ	1/4
	6	2S3	TGTGCCAGC	AGCCAAGACCCCTCAGGTCATGAGCAG	TATTTTGGC	SQDPSGHEQ	1/4
	6	2S1	TGTGCCAGC	AGCTTAGATAGCGGAGAAGGACCCTCTACGAGCAG	TTCTTCGGG	SLDSGEGPSYEQ	1/4
	6	2S5	TGTGCCAGC	AGCCTAAGAGGGAAAGACCAG	TACTTCGGG	SLRGKQDQ	1/4
SIL	8	2S5	TGTGCCAGC	TCGCCGCTAGCGGGGAAAGAGACCCAG	TACTTCGGG	SPLAGKETQ	1/10
	8	2S5	TGTGCCAGC	AGCCTCCTTGTGGGGGGCTTAAAGAGACCCAG	TACTTCGGG	SLLVGGLKETQ	4/10
	8	1S5	TGTGCCAGC	AGCTTCGGGGGACGGGCAATCAGCCCCAG	CATTTTGGT	SFGGTGNPQ	4/10
	8	2S5	TGTGCCAGC	GGCCTGGGGGGGGCGCAGACCCAG	TACTTCGGG	GLGGRQTQ	1/10
SIL	16	1S2	TGTGCCAGC	AGCCAAACAGGGGCTTCATGGCTAC	ACCTTCGGT	SQTGGLHGY	4/4
CD8 PBMC	16	2S2	TGTGCCAGC	AGCCAAAGTCTC	TTTTTTGGA	SQSLTGEL	1/7
	16	2S1	TGTGCCAGC	AGCCAAGGTGTTACAATGAGCAG	TTCTTCGGG	SQGGYNEQ	1/7
	16	2S1	TGTGCCAGC	AGCCAAGAGGTAGCCAG	TTCTTCGGG	SQEVAG	1/7
	16	2S5	TGTGCCAGC	AGCCAGGCGGGGGCGGTGCAGAGACCCAG	TACTTCGGG	SQAGGGAETQ	1/7
	16	2S3	TGTGCCAGC	AGCCCCGGGGAGTGTCTTCAGATACGCA	TATTTTGGC	SPGGVSSAYA	1/7
	16	1S2	TGTGCCAGC	AGCCAAGGGAACCTTATATGGCTACACCTT	ACCTTCGGT	SQGNLIWLHL	1/7
	16	2S3	TGTGCCAGT	AGTGGGACAGGGGGCGCAGATACGCATATT	TATTTTGGC	SGTGGADTHI	1/7

Sequences are available in GenBank under accession numbers Z95081–Z95112. AA, Amino acid. Redundant sequences, corresponding to clonal expansions, are in bold.

from immunoscope analysis of that subset (see Fig. 4, patient A, sample 1). In contrast, the BV16 PBMC clones sequenced were all different.

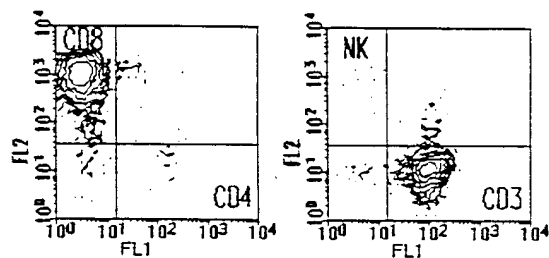
Thus, sequencing of the CDR3 regions confirmed the CDR3 dependence of the cutaneous T cell expansion.

Class I MHC-restricted cytotoxic recognition of HIV antigens by skin-derived CD8+ T cell lines. To test the ability of SILs to recognize and lyse target cells expressing HIV structural or regulatory protein-derived peptides, short-term CD8 T cell lines were generated from the SILs and PBMCs of patient A, and from the SILs of patient D. After allogeneic stimulation and in vitro expansion in IL-2-containing medium for 4 wk, cytofluorometric analysis showed that > 90% of SIL-derived cell lines exhibited a CD3+ CD8+ CD4− phenotype (Fig. 5). We investigated the cytotoxic activity of these lines against autologous or allogeneic B-LCL cells infected with vaccinia virus constructs expressing different HIV proteins. As shown in a previous preliminary report, the CD8 SIL-derived cell lines from patient A exhibited a strong lytic activity against

autologous targets expressing Gag and Pol gene products, whereas anti-Nef cytotoxic activities appeared marginal (17). In contrast, HIV-1 Nef was shown to be recognized predominantly by CTL lines originating from the PBMCs of the same patient (Table VI). SIL lines derived from patient D were predominantly Env-specific, although a weak Pol-specific lytic activity was also detected (Fig. 6).

The class I MHC restriction of the HIV-specific lysis mediated by cutaneous lymphocytes was investigated. CTL lines derived from the skin of patient A efficiently lysed Pol-expressing allogeneic targets which shared the patient's HLA-B8 antigen, whereas they did not kill HLA-B-mismatched B-LCL infected with VAC-POL (Fig. 6 A). In patient D, the SIL-derived CTL line was shown to be lytic against allogeneic Env-expressing B-LCL 2226 sharing the patient's HLA-B72 specificity (Fig. 6 B). This latter CTL line exerted no specific lysis when tested against the A23/B49 expressing B-LCL 2210, or when a totally class I-mismatched, DR11-expressing B-LCL was used as target.

A



B

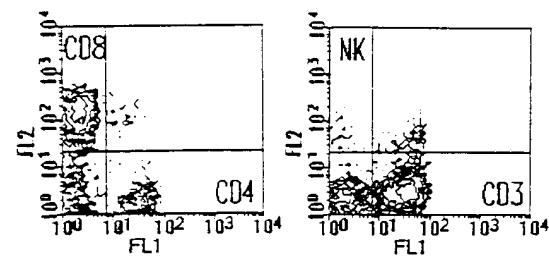


Figure 5. (A) Cytofluorometric analysis of the CTL line obtained from the SILs of patient A; 98% of the cells are CD3+ CD8+ and do not express natural killer (NK) cell markers (CD56 and CD16). (B) Analysis of PBMCs from patient B; 82% of the cells are CD8+, 10% are CD4+, and 7% bear natural killer (NK) cell markers.

Altogether, these results demonstrate that the CTL recognition of HIV gene products by SILs originating from patients A and D is restricted by HLA-B8 and HLA-B72 class I antigens, respectively.

In vitro-expanded SIL lines are representative of the skin infiltrate. To ascertain that functionally analyzed cell lines were representative of the skin infiltrate, we studied the TCRBV repertoire used by CTL lines generated from the SILs of patient A. As shown in Table VII, the cDNA sequence analysis of junctional regions expressed in the T cell lines was performed for the subsets that were not clonal in situ, i.e., BV2, BV6, and BV8. We were concerned that the polyclonal back-

Table VI. HIV-specific Cytotoxic Activities in CD8+ T Lymphocytes Derived from the PBMCs of Patient A

E/T ratio	Percentage of HIV-1-specific lysis*	
	HIV-1 Pol	HIV-1 Nef
30/1	15	55
10/1	2	38
3/1	5	22

The predominant anti-Nef activity detected in the blood-derived CTL line contrasts with the strong anti-Pol CTL activity of a CTL line derived from the skin of the same patient (see Fig. 6). *Effector cells were tested against autologous EBV-transformed cell lines infected with VAC-POL, VAC-NEF, and VAC-WT as control.

ground might have been amplified artificially in vitro in such cases. CDR3 length analysis (not shown) and CDR3 sequencing of the BV segments used predominantly by one short-term CTL line revealed redundant rearrangements identical to those overexpressed in situ.

Discussion

The data presented here provide evidence for the infiltration of HIV-specific CTLs in the skin of HIV-infected patients affected by a severe skin disorder occurring years after a primary HIV infection. Results from previous studies indicated that patients presenting with this HIV-related chronic skin disorder demonstrate infiltration of both the dermis and epidermis by activated CD8+ T cells (17). Although histological examination of the lesional skin may reveal in some cases a dense infiltrate, cytonuclear irregularities are not consistently found, and no aberrant expression of T cell antigens by SILs can be found. Furthermore, molecular genetic studies did not show evidence for a dominant clonal rearrangement of the TCR loci (16–18). These preliminary data argued against a neoplastic T cell disorder, and we therefore postulated that a CD8 cell-mediated immune response against HIV antigens could be involved in the pathogenesis of this syndrome.

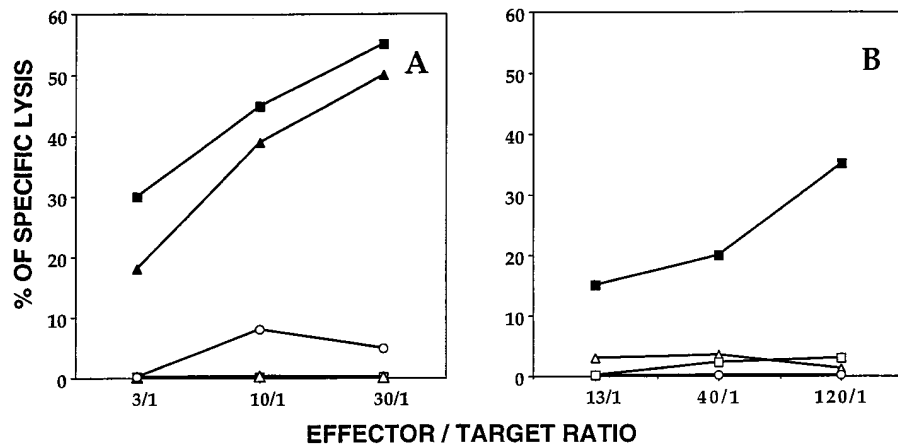


Figure 6. (A) The CD8 cell line derived from the SIL1 sample of patient A mediates specific and HLA-B8-restricted lysis of Pol-expressing target cells. Effector cells (HLA A1-A11/B8-B63/DR13-DR13) were tested against autologous (filled squares), HLA-A1/B8-sharing (filled triangles), HLA-A1-sharing (open squares), HLA-DR13-sharing (open triangles), and MHC-mismatched (open circles) (HLA A26(10)-A32/B55(22)-B44(12)/DR4-DR5) B-LCL target cells, all infected with VAC-POL. (B) The CD8 cell line from the SILs of patient D mediates specific and HLA-B72-restricted lysis of Env-expressing target cells. Effector cells (HLA-A23(9)-A/B49(21)-B72(70)/DR13-DR11) were tested against HLA-B72-sharing (filled squares), HLA-B49-sharing (open circles), HLA-A23-sharing (open triangles), and MHC-mismatched (open squares) B-LCL target cells, all infected with VAC-ENV.

ing (filled squares), HLA-B49-sharing (open circles), HLA-A23-sharing (open triangles), and MHC-mismatched (open squares) B-LCL target cells, all infected with VAC-ENV.

Table VII. Sequences of BV-BJ TCR Junctional Regions Cloned from PCR Products Obtained from a CD8 CTL Line Derived from the Lesional Skin of Patient A, and Predicted Amino Acid Sequences of the CDR3 Region

Source	BV	BJ	V β	CDR3 region	J β	CDR3 AA sequence	Frequency
SIL line	2	2S1	TGCAGTGTCT	AGAGATCACAGCGGGGGCTCTGGGAATGAGCAG	TTCTTCGGG	RDHSGASGNEQ	6/8
	2	2S1	TGCAGTGTCT	AGAGAACCACTAGCGGGAGGGCTATGGATGAGCAG	TTCTTCGGG	REPTSGRAMDEQ	1/8
	2	1S1	TGCAGTGTCT	AACTACAAGCCCGACAGGGAGGCACTGATGCT	TTCTTTGGA	NYKPRQGGTDA	1/8
SIL line	6	2S7	TGTGCCAGC	AGCTTAGGACAAGCCTACGAGCAG	TACTTCGGG	SLGQAYEQ	5/5
SIL line	8	2S5	TGTGCCAGC	AGCCTCCTTGTGGGGGGGCTTAAAGAGACCCAG	TACTTCGGG	SLLVGGLKETQ	2/4
	8	1S5	TGTGCCAGC	AGCTTCGGGGGACGGGCAATCAGCCCCAG	CATTTTGGT	SFGGTGNQPQ	2/4

Sequences are available in GenBank under accession numbers Z95081–Z95112. Sequences found previously in SILs before in vitro culture are in bold. AA, Amino acid.

In the first part of this report, we investigated in four patients the diversity of the TCRBV repertoires used by SILs and by CD8 PBMCs. When the repertoires from blood and lesional skin samples were compared within the same patient using semiquantitative BV-specific RT-PCR, striking differences were observed. Particular BV transcripts appeared to be relatively more abundant in samples derived from skin lesions. Although some BV subsets were found to be overexpressed in the SILs from several patients, such as BV2 in patients A and D and BV16 in patients A and B, no common BV expression profile was demonstrated in all patients, suggesting that the skin-homing CD8 expansion was not related to a common superantigenic stimulation. Furthermore, since the results from in situ immunostaining analysis using a restricted set of anti-BV mAbs provided results that were correlated with those obtained by PCR analysis, it is likely that the increased levels of particular BV transcripts do not simply reflect a specific activation, but also result from a preferential proliferation of CD8 T cells expressing these BV segments. Since all patients presented with a generalized erythroderma, normal skin was not available for comparative analysis from the same individual. However, the most represented BV subsets within the SILs from the patients were not predominant either in the normal skin from two HIV-seropositive individuals (Table IV) or in normal human skin as reported previously (35), indicating that a nonspecific expansion of skin-resident CD8 cells was unlikely to account for this cutaneous lymphoinfiltrative process.

We investigated more accurately the T cell diversity within the SILs and PBMCs of the four patients using the immunoscope approach. This method has been used previously to characterize in vivo the limited heterogeneity of the TCR β repertoire used in a CD8 cell-mediated primary immune response in mice (36), whereas in humans, a similar approach allowed the detection of oligoclonal patterns of HIV-specific CTLs in the spleens of HIV-infected patients (11). In the four patients studied here, immunoscope profiles of the most represented BV subsets in the SILs displayed oligoclonal profiles, usually clearly distinct from those obtained from blood. However, a few identical peaks were also found in the CD8 PBMCs, suggesting a recirculation of skin-homing CD8 cells (see Fig. 4, patient 4, BV4). The oligoclonal nature of the skin infiltration was further confirmed by sequencing of the TCR β junctional regions cloned from the SILs and from the PBMC compartments in patient A. Indeed, the redundant use of CDR3 amino acid sequences within the BV2, BV6, BV8, and BV16 subsets was highly suggestive of an antigen-driven ex-

pansion of CD8 cells bearing these BV segments in the skin lesions, and argues against a superantigenic stimulation (37). In HIV-infected patients, a restricted repertoire of BV segments has been documented in the HIV-specific CTL expansion in the blood after primary infection (5), and among the CD8⁺ T cell infiltration of the salivary glands in the HIV-related Sicca syndrome (38).

The potent HIV Gag- and Pol-specific lytic function exhibited by SILs from patient A when tested at low E/T ratio suggests a high frequency of HIV-specific CTL precursors within the skin infiltrate. The relatively lower level of Env-specific lysis displayed by the SILs from patient D is consistent with the decrease of HIV-specific CTL activity observed during the progression towards AIDS (39). Besides those quantitative differences, our results suggest that immunodominant HIV antigens differ from one patient to another. The comparative analysis of the TCRBV repertoire expressed in vivo and after in vitro expansion confirmed that CTL lines were representative of the cutaneous T cell infiltration and did not originate from contaminating blood lymphocytes. Most CDR3 rearrangements that were overrepresented in the SIL lines and in the skin infiltrate were not found among the cloned BV sequences originating from the corresponding CD8 PBMCs.

Altogether, TCR β repertoire studies and cytotoxicity assays strongly suggest that HIV-specific CTLs might be implicated in the pathogenesis of this inflammatory cutaneous disorder. Nevertheless, we cannot rule out the existence of additional CTL specificities in the skin infiltrate of the affected patients. In any case, our results raise the issue of the cellular target of the in vivo CTL response. An increasing body of evidence provided in vitro (40) and in vivo (41) indicate that epidermal Langerhans cells (LCs) can be productively infected by HIV-1. Like dendritic cells, which present antigen-derived peptides to CD8⁺ CTLs in a class I MHC-restricted fashion (42), LCs could play the role of antigen-presenting cells involved in the activation of CTLs during HIV infection. The detection of Pol-specific mRNA in the epidermal tissue sampled in patient A by the suction blister technique supports this latter hypothesis, whereas HIV RNA was not detected in the skin biopsies using in situ hybridization (data not shown). A preferential replication in LCs of a particular HIV-1 subtype might explain the predominant infiltration of the skin observed in these patients. However, such a mechanism is not supported by in situ immunohistochemical studies performed in patient A, showing no immunoreactivity of the skin biopsy with the anti-HIV-1 p24 mAb, whereas p24 antigen was detected in the

lymph node biopsy. Therefore, it appears that there was no preferential HIV replication in the skin of patient A. It is much more likely that the predominant location of the CD8 infiltrate in the skin is related to the preferential expression of CLA within the CD8 T cell subset, as described previously in CTCLs (43). However, these data do not rule out the pathogenic role of a low level of HIV replication in the skin of these patients, since a sustained production of HIV-derived peptides in dendritic cells might lead to the chronic activation of HIV-specific CTLs among the CLA⁺ cells that are homing in the skin. The question still remains as to how CLA is upregulated in vivo.

The spatial variability of the BV repertoire used by SILs derived from two patients suggests a compartmentalization of the immune response. Similar topographic changes in the TCRBV repertoire have been found in the CTL infiltration of the splenic white pulp of AIDS patients presenting with thrombocytopenia (11). Since a topographic heterogeneity of HIV quasispecies has also been evidenced in the normal skin of an HIV-infected patient (44), various HIV CTL epitopes might be expressed differentially in distinct skin areas (45). Thus, HIV variability could explain in part the heterogeneous local recruitment of T cells that we have documented. Studies are in progress to address this hypothesis.

Although specific CTLs are probably critical in the control of HIV spreading after primary infection, recent data indicate that tissue infiltration by virus-specific CTLs might also contribute to the immunopathogenesis of various sequelae emerging during the course of HIV infection. The presence of HIV-specific CTLs has been demonstrated in the lungs of patients with lymphocytic alveolitis (6) and in the splenic white pulp of patients with splenomegaly and thrombocytopenia (11). Our data corroborate the results of previous work on the skin eruption that follows experimental infection of macaque rhesus monkey with SIV. Viral RNA was not detected in the skin of the animals by in situ hybridization, but only by PCR (15), as in the case of patient A in our study. In the animal model, both dermis and epidermis are infiltrated by activated CD8 T cells exhibiting SIV Gag-, Env-, and Pol-specific lytic activity (15). Therefore, although the skin viral burden might not be very high in the SIV model, viral antigens can nevertheless be presented efficiently in the skin. Both studies support the view that the skin might be a major site of interaction between HIV and the CTL response. Transgenic mice containing an HIV-1 provirus show a tissue-dependent level of transgene expression, the skin being one of the preferential sites of HIV-1 mRNA expression (46). Thus, organ-specific manifestations of AIDS might be explained partly by the existence of tissue-specific factors important in HIV-1 transcriptional and posttranscriptional regulation that could qualitatively affect the local expression of antigens.

In a recent study, HIV-specific CTLs could be detected in the genital mucosa of 63% of the HIV-infected women tested (47). It remains to be determined whether HIV-specific CTLs also infiltrate the normal skin of HIV-infected patients, where they might be involved on a regular basis in the elimination of infected LCs and skin-homing CD4 T cells.

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