Autoreactive Cytotoxic T Lymphocytes in Human Immunodeficiency Virus Type 1–Infected Subjects

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Summary

A subtractive analysis of peptides eluted from major histocompatibility complex (MHC) class I human histocompatibility leukocyte antigen (HLA)-A2.1 molecules purified from either human immunodeficiency virus type-1 (HIV-1)–infected or uninfected cells was performed using micro high-performance liquid chromatography and mass spectrometry. Three peptides unique to infected cells were identified and found to derive from a single protein, human vinculin, a structural protein not known to be involved in viral pathogenesis. Molecular and cytofluorometric analyses revealed vinculin mRNA and vinculin protein overexpression in B and T lymphocytes from HIV-1–infected individuals. Vinculin peptide-specific CTL activity was readily elicited from peripheral blood lymphocytes of the majority of HLA-A2.1$^+$, HIV$^+$ patients tested. Our observations suggest that atypical vinculin expression and MHC class I–mediated presentation of vinculin-derived peptides accompany HIV infection of lymphoid cells in vivo, with a resultant induction of antivinculin CTL in a significant portion of HIV$^+$ (HLA-A2.1$^+$) individuals.

CTL (CD8$^+$) recognize antigenic peptides complexed with MHC class I molecules on the surface of target cells. Cytotoxic T cells play a crucial role in the protective immune response to numerous experimental models of virus infection and disease (1, 2). There also is strong evidence that HIV-1–specific CTL play a pivotal role in controlling HIV in vivo. Vigorous HIV-1–specific CTL responses have been detected in infected asymptomatic individuals, and these responses subsequently decline with disease progression (3). Furthermore, recent studies indicated a correlation between the presence of CTL immunity and the ability of some of these individuals to control virus spread and replication and to become long-term survivors of HIV infection (4–6).

Conversely, a possible role for virus-specific CTL in HIV-induced immunopathology has also been postulated. Adoptive transfer of a HIV-specific CTL clone to an AIDS patient resulted in the selection for mutant HIV variants and disease progression (7). In addition, very high viral replication in the presence of a potent CTL response could also lead to T cell–mediated immunopathological damage, as observed in some model infections with noncytopathic viruses (8).

The identification of naturally processed, class I–presented peptides unique to HIV-infected cells could provide information regarding HIV-related epitopes and the functional role of CTL in HIV infection and disease. To this end, in this study we performed a subtractive analysis of peptides eluted from MHC class I HLA-A2.1 molecules purified from either HIV-1–infected or uninfected cells by micro-high HPLC and mass spectrometry (MS)[1]. Class I–bound peptides arising as a result of HIV infection would be expected to derive from viral proteins but might also derive from endogenous (cellular) proteins. The application of this very sensitive new technology allowed us to identify the

Contributions of F. di Marzo Veronese, D. Arnott, and V. Barnaba were equivalent and the order of their names should be considered arbitrary.

[1] *Abbreviations used in this paper:* ESI-TMS, electrospray-ionization/triple quadruple mass spectrometry; MS, mass spectometry; m/z, charge/mass; RP, reverse phase.

most highly represented peptides in the infected cells without biasing the search towards a specific gene product.

Materials and Methods

Cell Lines and Viruses. The EBV-B lymphoblastoid cell line designated AA2 is highly permissive for HIV-1 infection (9) and expresses HLA-A2.1. We chronically infected the AA2 cell line with HIV-1 strain IIIB. The cell line was tested for expression of HIV-1 proteins by p24 antigen capture assay and immunoprecipitation of lysates prepared from metabolically [^{35}S]cysteine–labeled cells. The antigen capture assay was performed according to the instructions of a commercially available kit from Cellular Products (Buffalo, NY) and revealed specific release of ~100 ng of HIV-1 p24/ml in the supernatant of the infected cells. The immunoprecipitation was performed with a serum from a HIV-1–positive subject and unequivocally demonstrated specific and abundant expression of HIV-1 proteins. Individual clones were derived from the bulk-infected cells by limiting dilution and were monitored for viral protein expression by p24 antigen capture assay and by indirect immunofluorescence. One of the clones, showing evidence of uniform and abundant viral envelope expression in all cells by immunofluorescence, was designated AA2-IIIB and selected for large scale expansion. JY is an EBV-B lymphoblastoid cell line also expressing HLA-A2.1. Jurkat is a T cell line derived from acute T cell leukemia. Jurkat A2 was derived from Jurkat upon transfection of the HLA-A2.1 gene.

Extraction of MHC-bound Peptides. Each batch of peptides was the product of a cell pellet from 10 liters of cells containing ~5 × 10^9 cells. Purification of MHC and elution of peptides was carried out according to previously described procedures (10). Soluble proteins from the cell lysate were passed over GAP-A3 (irrelevant antibody) and BB7.2 (anti-HLA-A2.1) immunoaffinity columns. Peptides were eluted with 0.2N acetic acid, pH 2.7, further acidified with glacial acetic acid to pH 2.1, and collected by centrifugation through a 5-kD filter. The resulting fractions were concentrated by vacuum centrifugation and screened by MS. Those fractions that contained peptides were pooled together. Typically, three preparations from 10-liter cell suspensions were extracted and pooled for analysis.

Reduction and Carboxyamidation of Cys-containing Peptides. Reduction of disulfide bonds and derivatization of free SH groups were performed as follows. Peptide samples were reduced in volume to 1–2 μl by vacuum centrifugation. Dithiothreitol (30 nmol in 5 μl degassed Tris-buffer, 500 mM, pH 8) was added and the sample vortexed. The solution was incubated under argon at 37°C for 1 h. To the reduced peptides, iodoacetamide (70 nmol in 5 μl H_2O) was added. The mixture was vortexed and incubated in the dark, under argon, at 37°C for 1 h. The resulting derivative was analyzed without further treatment, by microcapillary HPLC-electrospray ionization/triple quadruple mass spectrometry (ESI-MS).

Reverse-phase–HPLC Separations. Fractionation of the peptide extracts was performed on a separations system (model 130A; Applied Biosystems, Foster City, CA). Peptide extracts were injected in 100 μl, 5% acetic acid solutions onto a narrow bore, reversed-phase column (RP-18 Spheri-5, 2.1 mm × 3 cm), and eluted at 200 μl/minutes over 40 min by a linear gradient of 0–60% acetonitrile (0.085% TFA) in 0.1% TFA. Fractions were manually collected into 0.65 μl siliconized tubes at 1-min intervals and immediately frozen with dry ice. Column effluent was monitored at 214 nm. Care was taken to insure that each fractionation was carried out under as nearly identical conditions as possible. HPLC fractions were concentrated by vacuum centrifugation to remove the acetonitrile and TFA before mass spectral analysis.

Microcapillary RP-HPLC Separations. Samples were introduced to the mass spectrometer by microcapillary RP-HPLC. Samples were loaded hydrostatically onto a 40-cm length of fused silica capillary (75 μm inside diameter [i.d.], 180 μm outside diameter) packed with 10–15 cm of POROS II/RH RP beads. The amount of sample loaded was determined by measuring the volume of solvent displaced from the column with a 1–5-μl disposable pipette. Peptides were eluted by a 10-min gradient of 0–80% acetonitrile in 0.1 M acetic acid from a dual syringe pump (model 140A; Applied Biosystems). A precolumn split of the mobile phase (flowing at 200 μl/min) was used to obtain column flow rates of 0.2–0.5 μl/min. A 50 μm-i.d. restriction capillary was used to control the back pressure of the system.

ESI-MS. Mass spectra were recorded on a triple quadrupole mass spectrometer (model TSQ-70; Finnigan, San José, CA) that had been upgraded with the electronics and data system of TSQ-700, and the Finnigan API ion source. A coaxial sheath liquid of 3:1 methanol/0.1% acetic acid was employed. The spray was assisted by coaxial N_2 gas flow from sheath and auxiliary gas ports. Analytes were dissolved and transmitted to the first quadrupole by passage through a heated capillary and differentially pumped octapole lens region.

Synthetic Peptides. Synthetic peptides based on HIV protein sequences were provided by the Cytel Corporation. Other peptides were synthesized using solid-phase Fmoc chemistry on Wang resins with a multiple peptide synthesizer (model AMS 422; Gilson, Middletown, WI).

RNA Extraction and Dot Blot Analysis. Total RNA was extracted from 10^6 cells by the protocol supplied with the RNA Micro-Isolation Kit (Stratagene Inc., La Jolla, CA). Duplicate aliquots of ~1 μg of each sample were transferred to nitrocellulose membranes using a dot blot apparatus (Bio-Rad Laboratories, Hercules, CA). Membranes were incubated at 42°C with 10^6 cpm/ml of either a 50-mer vinculin oligonucleotide or a glyceraldehyde phosphate dehydrogenase (GAPDH) probe, washed, and then exposed to either x-ray film or the imaging screen of a PhosphorImager 425 (Molecular Dynamics, Sunnyvale, CA).

Flow Cytometry. Cells were fixed with 4% paraformaldehyde in PBS for 5 min at room temperature (RT), and washed three times with 50 mM NH_4Cl in PBS and two times with cold PBS. Cells were permeabilized in 100 μl of 0.6% *n*-octyl-β-D-glucopyranoside for 5 min at RT and washed two times in PBS. Cells were then incubated with antivinculin IgG1 mAb (Chemicon International Inc., Temecula, CA) for 45 min at RT, washed and stained with a PE-labeled anti-IgG1 antibody (Southern Biotechnologies, Birmingham, AL). After further extensive washings, cells were incubated with either anti-CD3-FITC IgG2a mAb (OKT3-FITC; Ortho Diagnostics, Raritan, NJ) or anti-CD20-FITC IgG2b mAb (OKT20-FITC) for 30 min at 4°C. Finally, cells were washed and cell fluorescence was analyzed with the FACScan® flow cytometer (Becton Dickinson & Co., Mountain View, CA).

Generation of CTL Lines. PBMC (4–5 × 10^6) were incubated in RPMI containing 1% human AB serum for 90 min at 37°C and 5% CO_2. The nonadherent cells (PBL) were removed and the adherent fraction was pulsed with 100 μg/ml of synthetic peptide overnight and used as a source of APC. APC were then extensively washed and incubated with 1.5 X 10^6 responding PBL. After 5 d of culture, 10 U/ml rIL-2 (Proleukin, Emeryville, CA) was added and 2 d later, viable cells from each culture were

tested in a conventional 6-h cytotoxicity assay. Peptide-specific cultures were stimulated further with peptide-pulsed irradiated (3,000 rad) autologous PHA–T cell blasts used as APC, expanded with rIL-2, and tested for their ability to recognize endogenously synthesized antigen in infected target cells. PBL-derived from random-selected healthy subjects were primed in vitro by weekly restimulation with autologous peptide-pulsed APC and expanded with rIL2. After five to six rounds of restimulation, the cultures were analyzed for peptide-specific CTL activity and those found to be peptide-specific were used for the generation of CTL clones.

Generation of CTL Clones. T cells clones were isolated and maintained as previously described (11). Briefly, peptide-specific CTL lines were cloned by limiting dilution at 0.3 cells/well into 96-well U-bottom plates in the presence of 0.5 μg/ml PHA (PHA-P; Wellcome, Beckenham, UK), 50 U/ml rIL-2, and irradiated, allogeneic feeder cells. After 2–3 wk, cell growth was detected using an inverted microscope and growing cultures were tested for their ability to kill peptide-pulsed ^{51}Cr-labeled target cells. Peptide-specific $CD8^+$ CTL clones were then expanded in rIL-2–containing medium and maintained in culture with 2-wk cycles of restimulation with PHA and allogeneic APC.

Results and Discussion

Given the prevalent expression of the class I allele HLA-A2.1 (~46% among Caucasians) (12), we undertook a subtractive analysis of peptides extracted from HLA-A2.1 molecules purified from a B cell line that was either uninfected or infected with HIV_{IIIB} in vitro. We carried out our experiments using B cells since the peptides presented by HLA-A2.1 on B-lymphoblastoid cells have been previously characterized (10). Although B cells do not represent the primary

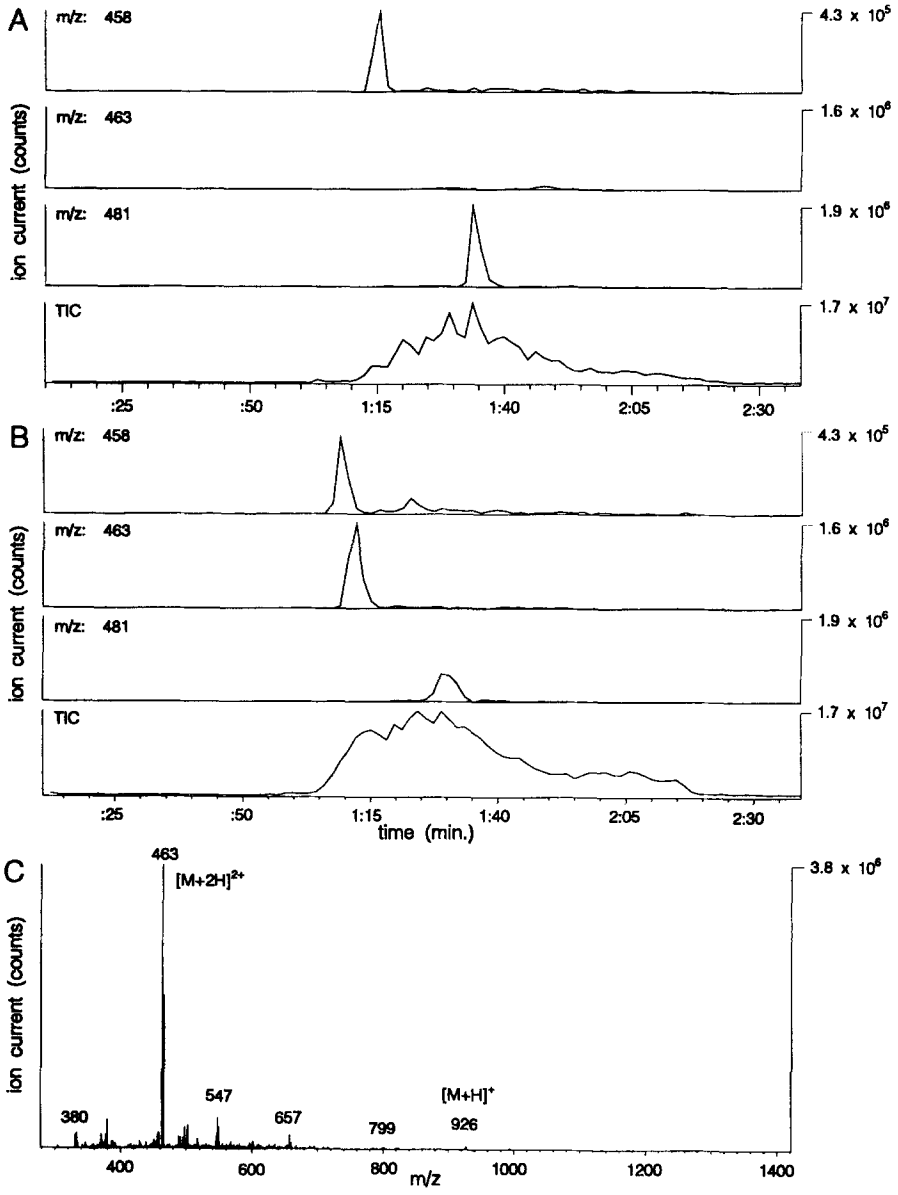

Figure 1. Ion chromatograms of peptides extracted from HLA-A2.1 molecules. HLA-A2.1 molecules were purified from lysates of AA2 and AA2-IIIB cells by immunoaffinity chromatography following described procedures (10). Shown is HPLC fraction 15 of uninfected (*A*) and HIV-infected (*B*) AA2 cells, with the mass spectrum (*C*) of a peptide unique to infected cells. The fractions containing peptides from three preparations were pooled and concentrated by vacuum centrifugation. Peptides were fractionated by HPLC on a 2.1 mm × 3 cm C18 column (ABI). A 40-min binary gradient at 200 μl/min of 0–60% solvent B was used to elute the peptides, with fractions collected at 1-min intervals. Solvent A was H_2O and 0.1% TFA; solvent B was 60% acetonitrile, 30% H_2O, 0.085% TFA. Fractions were concentrated and aliquots corresponding to 1.5×10^9 cells analyzed by capillary LC-MS as described previously (10). A spectrum covering the mass range 300–1,400 was acquired every 1.5 s. Total ion currents were obtained by summing the signal from every mass in each spectrum. The extracted ion currents represent the signal in each spectrum due to ions of a particular m/z.

target of HIV, B cell infection by HIV has been observed both in vitro (13, 14) and in vivo (15). Peptides were analyzed using the technique of capillary HPLC coupled with ESI-TMS (10).

Peptides associated with HLA-A2.1 molecules were purified from both control and 100% HIV-1–infected clonal populations of AA2 cells. The extracted peptides were fractionated by RP-HPLC. Aliquots of each fraction were rechromatographed by capillary RP-HPLC, and the resultant fractions were then directly analyzed by ESI-TMS. At every stage of the procedure, samples from control and HIV-infected cells were treated identically. The mass spectra of each HPLC fraction from infected cells were compared, peptide by peptide, to the mass spectra of the corresponding HPLC fraction from control cells. Where peptides appeared to be unique to either sample, adjacent fractions were also examined to determine whether or not the disparity was simply due to a shift in retention time.

An example of this approach is illustrated in Fig. 1, in which peptides from fraction 15 of control (Fig. 1 *A*) and HIV-infected (Fig. 1 *B*) cells were compared. The bottom panels of Fig. 1, *A* and *B* show the total ion chromatograms for each run; mass spectra were recorded every 1.5 s. The upper panels represent the elution of peptides with particular charge/mass (m/z) values. Virtually all of the abundant peptide ions identified from the control sample were also present in the sample from HIV-infected cells. Peptides with m/z 458 and 481 are present in both samples in similar proportions and were used as markers. The peptide with m/z 463, which elutes in the interval between these markers, is present in only the sample from infected cells. The summed mass spectra (Fig. 1 *C*) reveal that this unique peptide is doubly charged and has a nominal molecular mass of 925 daltons.

Application of this method revealed three peptides that appeared only in the samples derived from HIV-infected cells (Table 1). The sequences of these peptides were determined by collision-activated dissociation (CAD) on the triple quadrupole mass spectrometer. Each peptide is nine residues long and exhibits the HLA-A2.1–binding motif (10). Synthetic versions of peptides 1 and 2 displayed moderate affinity for HLA-A2.1 in an in vitro peptide–binding assay (16) (Table 1). Protein sequence databases were searched to identify the protein precursors of these peptides. The three peptide sequences were found to occur within a single cellular protein, human vinculin. Vinculin, a cytoskeletal protein, is an essential component of cell–extracellular matrix adhesions involving integrins (focal adhesions) and cell–cell adhesions involving cadherins (adherens junctions) (17). Peptides 1, 2, and 3 were redesignated according to their corresponding positions in the vinculin protein sequence (18) as $Vinc_{(359-367)}$, $Vinc_{(823-831)}$, and $Vinc_{(983-991)}$, respectively. The third peptide is unusual in that it contains a modified residue at position 3. Reduction and carboxyamidation of this peptide results in a loss of 45 daltons, consistent with the release of an unknown group with a mass of 102 daltons, followed by addition of the 57-dalton accompanying carboxyamidation of one Cys, suggesting that this residue is Cys modified through a disulfide linkage. It is not known whether this modification preceded antigen processing or was introduced during peptide extraction and purification. The third peptide exactly matches residues 983-991 of vinculin, providing that Cys is assigned to peptide position 3, corresponding to Cys_{985} in the published sequence (18), in accord with our interpretation of the CAD data.

Surprisingly, despite readily demonstrable synthesis of HIV proteins in AA2-IIIB cells, our analyses failed to identify HIV-derived peptides in eluates prepared from infected cells, either by subtractive analysis or through a direct search aided by a large panel of synthetic HIV peptides selected for their ability to bind to HLA-A2.1. Peptides that could be derived from HIV proteins in regions that are conserved among many viral strains were synthesized and tested for HLA-A2.1 binding using the quantitative, solu-

Table 1. *Peptides Extracted from HLA-A2.1 Molecules Uniquely Associated with HIV-infected AA2 cells (AA2-IIIB)*

Peptide	[M+H] (m/z)	Yield (fmol) AA2-IIIB	Peptide sequence	Vinculin sequence	HLA-A2.1 binding IC_{50}
					nM
1	914	140	GXDVXTAKV	GLDVLTAKV (358-366)	132
2	925	380	RXXGAVAKV	RILGAVAKV (823-831)	91
3	1,160	200	QVZERXPTX	QVCERIPTI (983-991)	ND

Aliquots corresponding to 1.5×10^9 cells were analyzed by capillary HPLC–ESI-MS. Yields are based on comparisons with a peptide standard. No discrete peaks corresponding to peptides 1–3 were discerned in AA2 (uninfected) eluates. Unresolved, low abundance peptides in these complex mixtures produced background signals of 1–10 fmol, establishing our threshold of detection. Amino acids are shown in single letter code: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr. The letter Z represents Cys modified by an unknown group with a molecular mass of 102. X is used to designate either Leu or Ile, which are not distinguishable by tandem MS. All three peptides were observed as the doubly charged ions $[M + 2H]^{2+}$ at one half of the m/z values shown. Also shown are the concentrations of peptide (IC_{50} values), which produced half-maximal inhibition of binding of a radiolabeled standard peptide to HLA-A2.1 molecules in an in vitro–binding assay.

ble MHC assay (16). A total of 68 peptides, including previously described epitopes, were identified that bound to HLA-A2.1 with various affinities; infected B cell class I preparations were analyzed for the presence of the 36 strongest binding candidates. Searches were based on the retention times of the synthetic peptides. Each synthetic peptide was screened by ESI-TMS to verify its molecular mass and to determine its characteristic charge state. An aliquot of each synthetic peptide was doped into a preparation of B cell peptides, and the resulting mixture was fractionated by RP-HPLC. Capillary HPLC–ESI-TMS was used to screen each fraction, and the synthetic peptides were identified on the basis of mass, charge state, and their absence in preparations that had not been doped. A list was thus compiled that located the HPLC fraction in which each HIV peptide was expected to be found, and the HPLC fractions of infected cell peptides were searched accordingly. For example, a peptide with a molecular weight of 1,012 was found in HPLC fraction 17 in the doped sample; analysis of fraction 17 of the HIV-infected sample revealed no corresponding peptide with the correct mass and charge state. The neighboring fractions were also analyzed for the peptide in case the HPLC separations were not completely congruent. No match was found in these fractions either. Several possible matches were generated by this method, but in each case the CAD spectrum of the candidate did not match that of the synthetic peptide. When searches for each of the 36 peptides proved unsuccessful, additional searches were initiated for the expanded set of peptides on the basis of molecular mass alone, but again no positive matches were found for these peptides. One obvious explanation for this result is that HLA-A2.1–associated HIV peptides are inefficiently processed and presented, at least in AA2-IIIB cells, and as a consequence are difficult to detect by ESI-TMS. The possibility remains, however, that the use of different conditions or cells might allow direct identification of HIV-derived peptides presented by infected cells.

The identification of vinculin-derived peptides uniquely associated with HLA-A2.1 molecules from infected cells suggested that vinculin might be overexpressed as a result of viral infection. To test this hypothesis, we performed dot blots (Fig. 2 *A*) with a vinculin oligonucleotide probe on total cellular RNA from AA2 and AA2-IIIB cells. In repeated experiments, strong signals were observed for infected cells, whereas vinculin mRNA expression was faint to undetectable in uninfected cells. Northern blot analysis confirmed that the signal observed for AA2-IIIB cells arose from a single intense band corresponding to the expected size of vinculin mRNA (data not shown). [^{35}S]Cysteine metabolic labeling of uninfected and infected cells followed by immunoprecipitation with vinculin-specific mAbs confirmed overexpression of vinculin at the protein level as well (data not shown). Dot blot analysis was additionally performed for total RNA from PBMC from HLA-A2.1$^+$, HIV-1–infected subjects in the symptomatic phase of AIDS, using PBMC from HLA-A2.1$^+$ healthy subjects as controls. Elevated vinculin levels were observed for each HIV$^+$ individual tested, indicating that the vinculin overexpression observed in AA2 cells upon in vitro infection reflects a more general phenomenon occurring in vivo. When PBMC were separated into adherent and nonadherent (PBL) fractions and similarly analyzed, increased vinculin expression was observed mainly in the PBL fraction, comprised mostly of T and B lymphocytes (Fig. 2 *B*). Moreover, flow cytometric analysis by two-color staining of PBL from HIV-1–infected subjects confirmed the increased expression of endocellular vinculin in a fraction of both the B and T cell populations (Fig. 2 *C*).

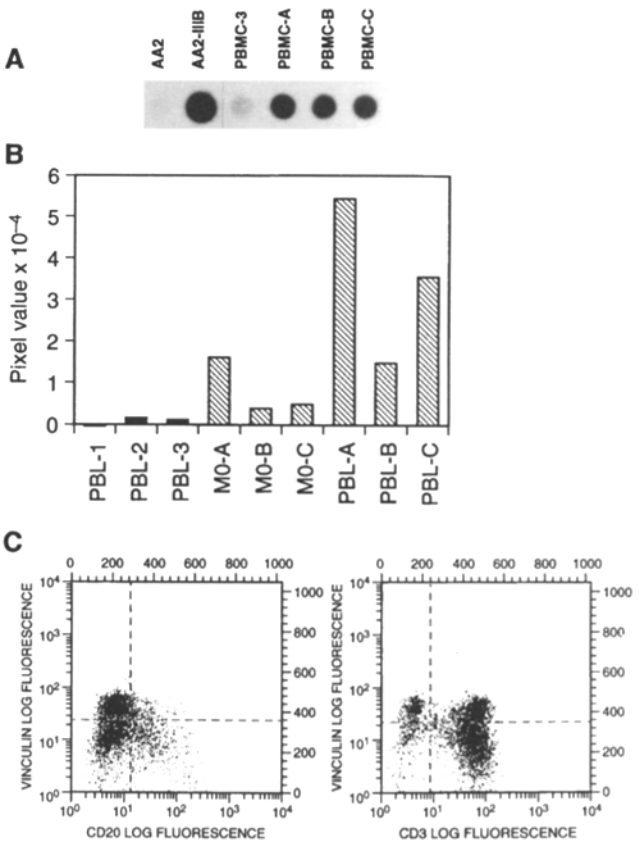

Figure 2. Expression of vinculin by dot blot analysis of mRNA levels (*A* and *B*) and by immunofluorescence labeling followed by flow cytometry (*C*). (*A*) Autoradiogram showing vinculin mRNA levels in HIV_{IIIB}-infected AA2 cells and PBMC from HIV$^+$ patients (A, B, and C) compared to uninfected AA2 cells and PBMC from an HIV$^-$ donor (donor 3). (*B*) Vinculin mRNA expression quantitated with a PhosphorImager. Pixel values were normalized to GAPDH values and are plotted for PBL from HIV$^-$ donors (PBL *1*, *2*, and *3*) and adherent (Mø) and nonadherent (PBL) fractions of PBMC from HIV$^+$ patients A, B, and C. (*C*) Two-color staining of vinculin and either CD20$^+$ B or CD3$^+$ T lymphocytes from a representative HIV-1$^+$ patient. The ordinate represents intracytosolic vinculin staining and the abscissa represents either membrane CD20 or CD3 expression. Intracellular vinculin expression was detectable in B and T cells of 8 out of 10 HIV-infected patients tested, but in 0 out of 10 healthy individuals.

To determine whether vinculin-derived peptides are immunogenic for CTL responses, two different approaches were followed. In the first approach, we sought to elicit CTL responses in PBL derived from HIV-1$^+$ patients or healthy donors after a single in vitro stimulation with autologous

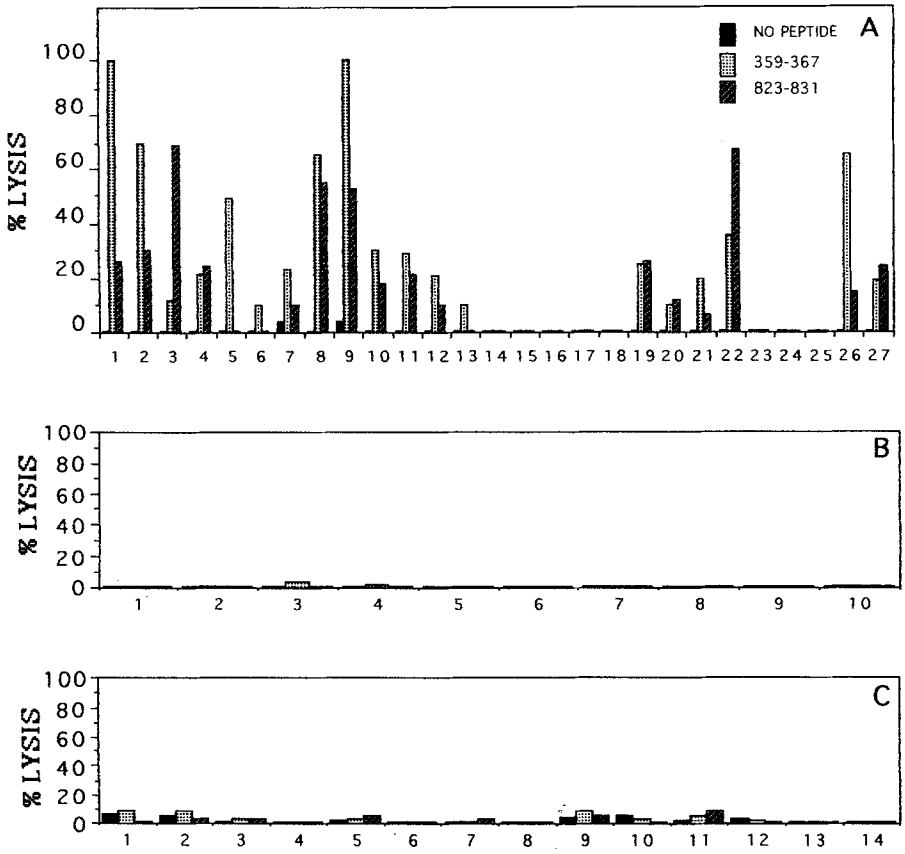

Figure 3. Vinculin-specific CTL response in patients with HIV infection. PBL derived from HLA-A2.1$^+$ (*A*) or HLA-A2.1$^-$ (*B*) HIV$^+$ patients, or from healthy subjects (*C*), were incubated with autologous adherent cells that previously were pulsed with 100 μg/ml of either $Vinc_{(359-367)}$ or $Vinc_{(823-831)}$ peptides. After 5 d, 10 U/ml rIL-2 was added and after a further 2 d, the cultures generated from HLA-A2.1$^+$ PBL were individually tested for specific cytotoxicity against peptide-pulsed (10 μg/ml) or untreated HLA-A2.1$^+$ cell lines JY and AA2 at an E/T ratio of 50:1. Cultures derived from HLA-A2.1$^-$ individuals were tested against autologous PHA-activated T cell blasts. Values for percent specific lysis reflect the mean of triplicate determinations. HLA-A2$^+$/HIV$^+$ patients (*A*) belonged to either the A1 group (patients *1–18*), A2 group (patients *19–23*), B2 group (patients *24* and *25*), or to the C3 group (patients *26* and *27*), according to the Centers for Disease Control classification scheme. All HLA-A2$^-$ patients (*B*) belonged to the A1 group. Healthy subjects (*C*) were negative for HIV, heapatitis B virus, and hepatitis C virus serological markers.

peptide-pulsed adherent monocytes (APC) (Fig. 3). CTL that lysed target cells pulsed with vinculin peptides were elicited in vitro after a single stimulation from PBL of HLA-A2.1$^+$ patients, but not from PBL of HLA-A2.1$^-$ patients or HLA-A2.1$^+$ normal donors, suggesting that CTL from HLA-A2.1$^+$ patients were vinculin primed in vivo. Moreover, 6 out of 11 long-term CTL lines, expanded from randomly selected vinculin-specific cultures of HIV patients by a second restimulation with peptide-pulsed APC and IL-2, killed not only peptide-sensitized target cells in a class I–restricted manner but also HIV-1–infected AA2 cells (Fig. 4 *A*). In the second approach, a protocol for in vitro priming of peptide-specific CTL, generated from PBL of healthy donors, was developed. After five to six rounds of stimulation with autologous peptide-pulsed APC, a peptide-specific CTL response was detectable in cultures from four out of nine healthy subjects tested, indicating that vinculin-specific CTL precusors are present in the normal T cell repertoire. As evident from a typical experiment shown in Fig. 4 *B*, three representative $Vinc_{(359-367)}$-specific CTL clones, generated from an in vitro peptide-primed T cell line, lysed both peptide-pulsed HLA-A2.1$^+$ target cells and AA2-IIIB cells. Jurkat cells expressing HLA A2.1, although productively infected with HIV-1_{IIIB}, did not overexpress vinculin as result of viral infection and were not susceptible to lysis by vinculin-specific CTL (Fig. 4 *B*). These results, together with the lack of any obvious homology between the vinculin-derived peptides described here and HIV-derived sequences, suggest that the self-reactive CTL observed among HIV$^+$ subjects arise as a consequence of vinculin overexpression and not through HIV/vinculin cross-reactivity.

The above findings provide in vivo correlates of our observations of AA2 cells infected with HIV in vitro. Overexpression of vinculin is directly observed in PBL from HIV$^+$ patients. Additionally, the finding of primed CTL, specific for the same vinculin peptides recovered from AA2-IIIB cells, in >50% of the HLA-A2.1$^+$, HIV$^+$ patients studied, strongly implies that HLA-A2.1–mediated presentation of vinculin-derived peptides occurs in vivo. Overexpression of cellular proteins upon HIV infection has been described previously by others (19–21) who have shown that dysregulated expression of cellular proteins may be driven by Tat-mediated transactivation of cellular promoters. Atypical (ectopic) cellular protein expression resulting in the induction of CTL has also been observed in a subpopulation of malignant melanoma patients (22, 23).

Recently, it has been demonstrated that presentation of self-antigens is induced by the HIV gp120-driven internalization of surface CD4 molecules, leading to enhanced CD4 processing and presentation of MHC class II/CD4 epitopes to autoreactive CD4-specific T helper cells (24). As described here, the creation of apparently novel class I–vinculin peptide complexes serves to trigger autoreactive CTL in a number of HIV$^+$ individuals. These results indicate that HIV infection upregulates the synthesis of vinculin in host

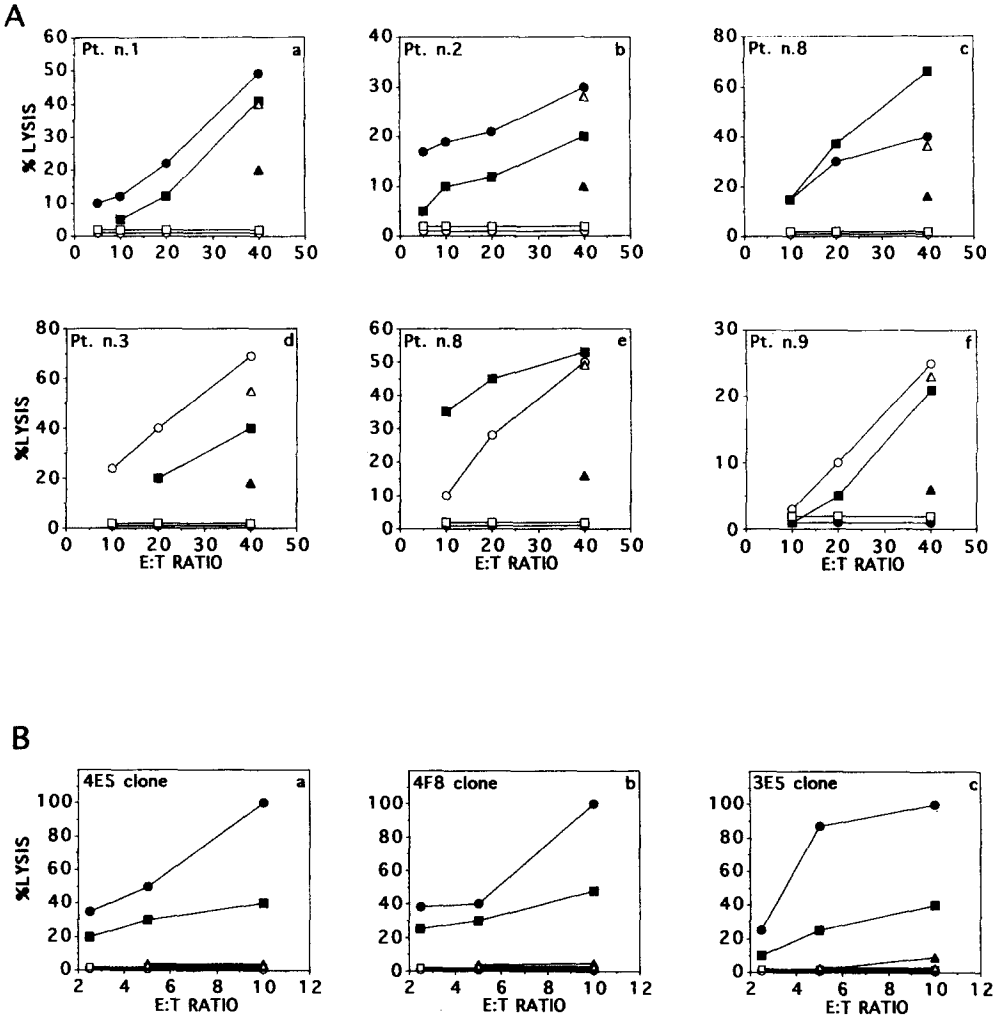

Figure 4. CTL raised against synthetic vinculin peptides recognize naturally processed peptides presented by HIV-infected cells, as well as peptide-pulsed target cells. (*A*) CTL derived from PBL of HIV$^+$ patients, selected for their capacity to lyse peptide-pulsed target cells, were expanded by a further restimulation with peptide-pulsed autologous APC and IL-2. (*a–c*) Representative $Vinc_{(359-367)}$- or (*d–f*) $Vinc_{(823-831)}$-specific CTL lines were tested for their capacity to lyse AA2 cells pulsed with either $Vinc_{(359-367)}$ (●) or $Vinc_{(823-831)}$ (○) and in the presence of anti-HLA-ABC mAb W6/32 (▲) or a pool of anti-HLA class-II mAbs (△). Shown also are the results obtained using HIV_{IIIB}-infected (■) or uninfected (□) AA2 cells as targets. (*B*) Three representative $Vinc_{(359-367)}$-specific CD8$^+$ T-cell clones (*a–c*) generated from in vitro–primed CTL isolated from healthy subjects, tested for their ability to lyse $Vinc_{(359-367)}$-pulsed HLA-A2$^+$ JY (●) or HLA-A2$^-$ HHK (○) target cells, HIV_{IIIB}-infected (■) or uninfected (□) AA2 cells, and HIV-1_{IIIB}-infected (▲) or uninfected (△) Jurkat HLA-A2.1$^+$ cells.

cells, allowing the generation of vinculin-derived epitopes in cytosolic processing compartments, and the priming of autoreactive vinculin-specific CTL responses. Moreover, the finding that primary vinculin-specific CTL responses can be generated in vitro from PBL of healthy subjects, after repeated stimulations with vinculin peptide–pulsed APC, suggests that these self-reactive CTL are present in the peripheral repertoire of normal individuals and become activated upon HIV infection. Self-reactivity in general is currently understood to arise by several mechanisms (25). In addition to a potentially important role in HIV pathogenesis, the present results obtained for vinculin provide, to our knowledge, the first demonstration of virus-dependent overexpression of a cellular protein implicated in the induction of self-reactive CTL in infected individuals.

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