

# Carboxyl-terminal and Central Regions of Human Immunodeficiency Virus-1 NEF Recognized by Cytotoxic T Lymphocytes from Lymphoid Organs

## An In Vitro Limiting Dilution Analysis

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### Abstract

Cytotoxic T lymphocytes (CTL) specific for human immunodeficiency virus (HIV) proteins have been analyzed in lymphoid organs from seropositive patients. Indeed, an active HIV replication coexists with a major CD8<sup>+</sup> lymphocytic infiltration in these organs. We have shown in a previous report that HIV-seropositive patients lungs were infiltrated by HIV specific CD8<sup>+</sup> lymphocytes. In the present report, we show that HIV-specific CTL responses can also be detected in lymph nodes and spleens, and were mainly directed against the ENV, GAG, and NEF HIV-1 proteins. The primary NEF-specific CTL responses were further characterized by epitope mapping. Determination of epitope-specific CTL frequencies were performed by limiting dilution analysis. Our results indicated that, in addition to the central region of NEF (AA66-148), a new immunodominant region is recognized by CTL. This region corresponds to the carboxyl-terminal domain of NEF (amino acids 182-206). AA182-206 is recognized in association with at least two common human histocompatibility leukocyte antigen (HLA) molecules (HLA-A1 and B8), with clonal frequencies of one CTL per 10<sup>-5</sup> to 10<sup>-6</sup> splenic lymphocytes. Our data indicate that lymphoid organs may represent a major reservoir for in vivo activated HIV-specific CTL. Furthermore, the carboxyl-terminal domain of NEF was found to be conserved among several HIV strains. Therefore, our finding is of interest for further HIV vaccines development. (*J. Clin. Invest.* 1992; 89:53-60.) Key words: epitopes • lungs • lymph nodes • spleens

### Introduction

Cytotoxic T lymphocytes (CTL)<sup>1</sup> play an important protective role during murine viral infections (1-3). Similarly, CTL could

be effective in the control of human immunodeficiency virus (HIV) infection in humans. This underlines the interest of studying cell-mediated immunity to HIV and identifying specific epitopes that stimulate CTL. Numerous reports have demonstrated the presence of HIV-protein-specific CTL in the peripheral blood from infected patients (4-7). In previous studies, we have evidenced tissular HIV-specific CTL in lungs of HIV-seropositive patients with lymphocytic alveolitis (8-10). In contrast, the function and the fine specificity for HIV of these CD8<sup>+</sup> cells infiltrating lymphoid organs have not yet been investigated, despite frequent reports of the coexistence of HIV particles and a massive CD8<sup>+</sup> cell infiltration within the germinal centers (11-13). This germinal center can thus be considered as a critical site for interaction between HIV and immunocompetent cells. Moreover, the in situ production of various cytokines, such as interleukin 6 (IL-6) or interferon (IFN), strongly suggests that CD8<sup>+</sup> cells present in the germinal centers might be involved in anti-HIV CTL responses (14).

The structural and regulatory proteins of HIV-1 are recognized by CTL, and their epitope mapping is currently under investigation (5, 15-19). Previous studies with murine CTL specific for adenovirus or cytomegalovirus early genes have shown efficient protection against viral infection (3, 20). Therefore, immune responses directed against early HIV regulatory proteins could represent efficient defenses against viruses. The strong immunogenicity of NEF for both B cells and CTL (21) could play a role in the in vivo control of HIV replication (22, 23). These data suggest that the determination of new consensus epitopes within this early regulatory protein should be relevant in view of vaccine development.

In the present study, we analyze the cytotoxic T cell responses specific for HIV proteins in lymphoid organs from seropositive patients, with special regard to specificities directed against the NEF early regulatory protein. The detection of CTL specific for HIV in primary assays contrasts with general observations for antiviral T cell responses and could be related to the abnormally high frequencies of in vivo differentiated effector CTL specific for ENV and GAG proteins (24). These properties allowed us to define new NEF epitopes which are recognized in primary polyclonal CTL responses. Furthermore, the relative frequencies of such epitope-specific primary CTL had been defined in limiting dilution analysis (LDA).

### Methods

#### Recombinant vaccinia viruses (RVV)

RVV expressing HIV viral gene products have been used to characterize virus-specific CTL. The viruses used were the wild-type strain Copenhagen (VAC-WT), and various recombinants encoding either the

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Received for publication 31 January 1991 and in revised form 13 September 1991.

1. Abbreviations used in this paper: AA, amino acid; CTL, cytotoxic T lymphocyte; E/T, effector/target ratio; ITP, idiopathic thrombopenic purpura; LDA, limiting dilution analysis; PGL, persistent generalized lymphadenopathy; RVV, recombinant vaccinia virus; VAC, vaccinia virus.

*J. Clin. Invest.*

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0021-9738/92/01/0053/08 \$2.00

Volume 89, January 1992, 53-60

Table I. NEF Peptides Synthesized from HIV-1 BRU Sequence

AA position	Sequences
4-18	K-W-S-K-S-S-V-V-G-W-P-T-V-R-E
10-25	V-V-G-W-P-T-V-R-E-R-M-R-R-A-E-P
25-39	P-A-A-D-G-V-G-A-A-S-R-D-L-E-K
37-50	L-E-K-H-G-A-I-T-S-S-N-T-A-A
46-60	S-N-T-A-A-T-N-A-A-C-A-W-L-E-A
54-67	A-C-A-W-L-E-A-Q-E-E-E-E-V-G
66-80	V-G-F-F-F-P-V-T-P-Q-V-P-L-R-P-M-T
79-94	M-T-Y-K-A-A-V-D-L-S-H-F-L-K-E-K
93-106	E-K-G-G-L-E-G-L-I-H-S-Q-R-R
100-114	L-I-H-S-Q-R-R-R-Q-D-I-L-D-L-W-I
113-128	W-I-Y-H-T-Q-G-Y-F-P-D-W-Q-N-Y-T
132-147	G-V-R-Y-P-L-T-F-G-W-C-Y-K-L-V-P
148-169	V-E-P-D-K-V-E-E-A-N-K-G
160-174	E-N-T-S-L-L-H-P-V-S-L-H-G-M-D
175-190	D-P-E-R-E-V-L-E-W-R-F-D-S-R-L-A
182-198	E-W-R-F-D-S-R-L-A-F-H-H-V-A-R-E-L
192-206	H-H-V-A-R-E-L-H-P-E-Y-F-K-N-C

ENV (VAC-ENV), GAG (VAC-GAG), NEF (VAC-NEF), VIF (VAC-VIF), or TAT (VAC-TAT) antigens of the HIV-1 BRU isolate (7, 22, 25, 26) (Transgène, Strasbourg, France).

### Peptides

A series of 17 peptides covering the NEF sequence of the HIV-1 BRU isolate has been synthesized by Neosystem, Strasbourg, France. These partially overlapping peptides contained 12-17 amino acids (AA) (Table I), and were supplied by the Agence Nationale de Recherche sur le SIDA, Paris. Lyophilized peptides were diluted in RPMI 1640 (Flow Laboratories, Inc., McLean, VA).

### Preparation of target cells

A panel of heterologous Epstein-Barr virus (EBV)-transformed B-cell lines were selected for HLA antigens matched with donors, and used as target cells. EBV-transformed B-cell lines were infected with the appropriate RVV at a multiplicity of infection of 20 plaque-forming units (PFU) per cell. The cells were then incubated at 37°C for 1 h 30 min, washed and suspended at 10<sup>5</sup> cells per ml in RPMI medium supplemented with 1% glutamine, 1% pyruvate, 2% penicillin-streptomycin, and 10% fetal calf serum (R-10%), for 14 h at 37°C before <sup>51</sup>Cr labeling. Alternatively, uninfected <sup>51</sup>Cr-labeled EBV cell lines were incubated with peptides at the final concentration of 4 µg/ml for 2 h at 37°C in a 96-well microtiter plates.

### Preparation of effector cells

**Lymphoid organs.** The lymphoid organs from four seropositive patients were manipulated immediately after ablation: two lymph nodes, for persistent generalized lymphadenopathy (PGL) with follicular hyperplasia; and two spleens, for idiopathic thrombopenic purpura (ITP). The cells were teased out and resuspended in sterile conditions in R-10%. Red blood cells were eliminated by subjecting the cell suspensions to Ficoll-Hypaque density gradient centrifugation. These effector cells were tested for primary cytolytic responses without in vitro stimulation. In one case, lymph node lymphocytes were set up in culture with autologous irradiated lymph node cells (5,000 rad) and the monoclonal antibody (MAb) anti-CD3 (a gift of D. Bourrel, Centre Regional de Transfusion Sanguine, Rennes, France). Continuous growing cell lines were established by addition of IL-2 (20 U/ml) every 3 d. The cells were tested 15 d after stimulation.

**Bronchoalveolar lavages.** Functional HIV-specific CTL were obtained by broncho-alveolar lavage from lungs of 16 seropositive patients with lymphocytic alveolitis as described previously (9).

### Human histocompatibility leukocyte antigen (HLA) typing

Class I HLA transplantation antigens were serologically identified using a standard complement-mediated microcytotoxicity assay.

### Cytotoxicity assays

Standard chromium release assays were performed as previously described (9). Target cells were labeled with 60 µCi/10<sup>6</sup> cells of Na<sub>2</sub> <sup>51</sup>CrO<sub>4</sub> (Commissariat d'Énergie Atomique, Saclay, France). Suspensions of 4 × 10<sup>3</sup> target cells were added in triplicate to round-bottom 96-well microtiter plates (Costar, Cambridge, MA) before addition of effector cells at various effector/target cell (E/T) ratio. After a 4-h incubation period at 37°C, the supernatant was collected and the chromium release was measured on a γ-counter. Spontaneous <sup>51</sup>Cr release values were 15-25% of total incorporated radioactivity. The percentage of specific <sup>51</sup>Cr release was calculated as followed: percentage = 1 - (experimental-spontaneous release/total radioactivity-spontaneous release).

### LDA

HIV-specific effector CTL relative frequencies were determined for various lymphocyte populations by LDA, as previously described (9). Briefly, 4 × 10<sup>3</sup> <sup>51</sup>Cr-labeled B-cell lines, preincubated with different peptides of NEF, were distributed in 96-well microtiter plates. Effector cells were then added at various dilutions ranging from 125 to 8,000 cells per well in a final volume of 150 µl. Each dilution was tested in 36 replicate wells. The plates were incubated at 37°C for 4 h and supernatants collected and counted for radioactivity. A well was considered as positive for the presence of cytotoxic effector cells if <sup>51</sup>Cr release exceeded by 3 SD the mean radioactivity of control wells containing target cells alone. Effector cell frequencies were estimated by Poisson distribution analysis from the relationship between the number of lymphocytes per well and the natural logarithm of fractions of negative cultures. Effector cells frequencies were determined by the statistical method of χ<sup>2</sup> minimization as described by Taswell (27) applying Poisson probability theory to the single-hit kinetic model.

### Results

**GAG-, ENV-, and NEF-specific CTL are present in lymphoid organs.** In order to investigate HIV-specific CTL responses in lymphoid organs with special regard to the NEF specificities, we analyzed T cell-mediated cytotoxicity directed against HIV proteins in spleen and lymph node cells harvested from patients with ITP or PGL.

Table II. Cytotoxic Activities of Splenic T Lymphocytes against HIV Proteins

Target proteins	Donor AB*		Donor BR		Control	
	70:1 <sup>†</sup>	8:1	70:1	8:1	70:1	8:1
ENV	13 <sup>‡</sup>	0	60	19	0	4
GAG	10	5	18	5	5	3
NEF	20	4	30	5	4	0
VIF	8	0	17	3	0	0
TAT	5	0	15	7	4	0
WT	5	5	18	3	0	0

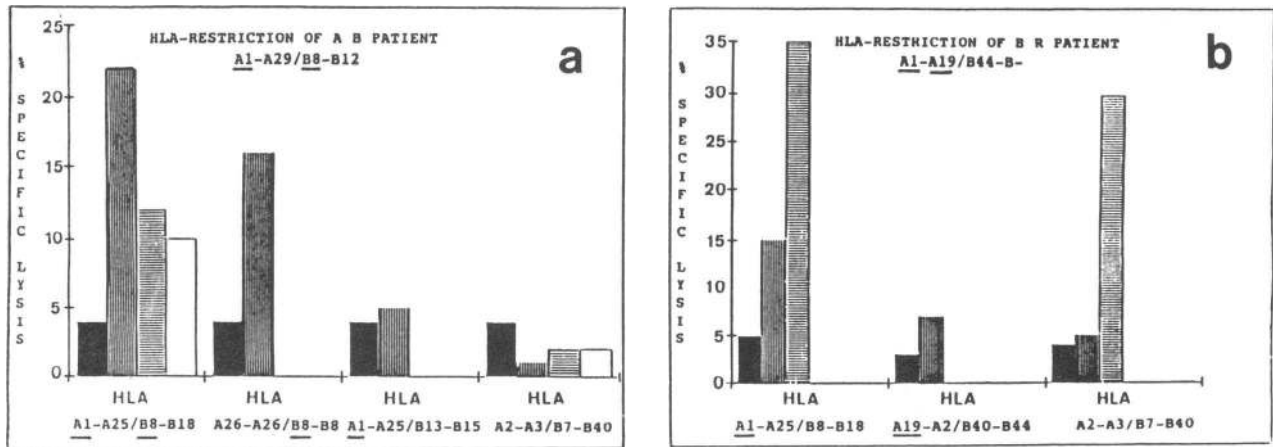
Donor AB: HLA A1-A29/B8-B12/DR7-DR53; target cells: HLA A1-A25/B8-B18/DR3-W6/CW7.

Donor BR: HLA A1-A19/B44-B<sup>-</sup>; target cells: HLA A1-A25/B8-B18/DR3-W6/CW7.

\* Effector cells were tested against HLA matched target cells.

<sup>†</sup> Effector to target cell ratio.

<sup>‡</sup> Percentage of specific chromium release.

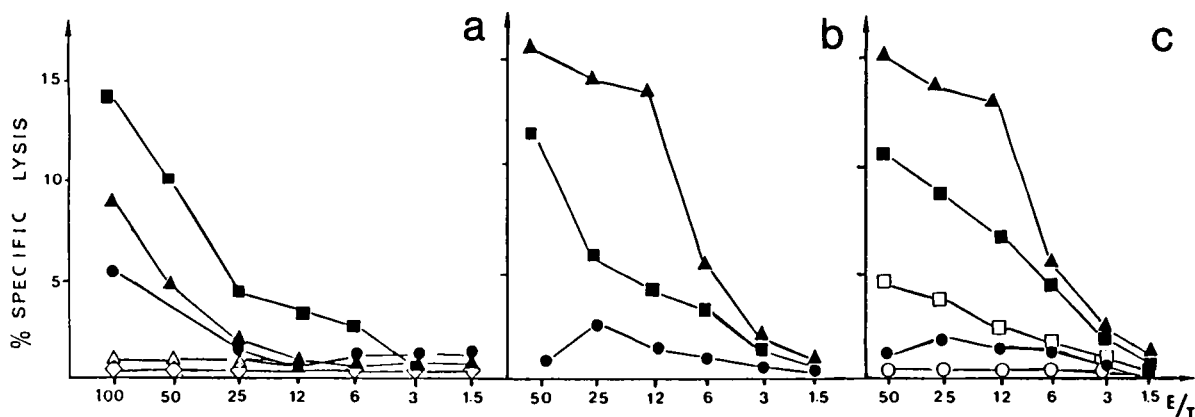


**Figure 1.** Primary splenic HIV-specific cytotoxic activities. Effector cells from patients AB (HLA-A1-A29/B8-B12/DR1-DR7-DR53) (a) and BR (HLA-A1-A19/B44-B<sup>-</sup>) (b) were tested at a 60:1 E/T ratio against allogeneic B cell lines infected with VAC-ENV (■), VAC-NEF (▲), VAC-GAG (□), VAC-WT (●). The HLA-class I molecules shared between the effector cells and the target cells are underlined. (a) HLA haplotypes of target cells: A1-A25/B8-B18/DR3-W6/CW7; A26-A26/B8-B8/DR2; A1-A25/B13-B15/DR1-DR4/CW1-CW6; A2-A3/B7-B40/DR4-DR8. (b) A1-A25/B8-B18/DR3-W6/CW7; A2-A19/B40-B44/BW4-BW6; A2-A3/B7-B40/DR4-DR8.

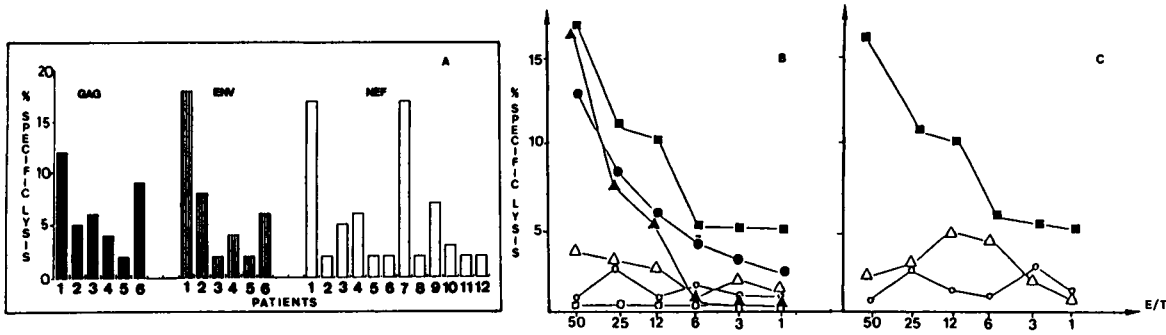
HIV-specific cytotoxic T cells were first studied in spleen cells from two HIV-seropositive patients with ITP. We used as target cells HLA-matched allogeneic EBV-transformed B-cell lines infected by RVV expressing a panel of HIV-1 proteins. As shown in Table II, we detected primary cytotoxic activities against ENV and NEF in both cases, and an anti-GAG activity in one case. We failed to detect any VIF or TAT protein-specific lysis. The primary cytotoxic responses specific for NEF were restricted by HLA class I molecules in both cases in that they were not detectable when tested against target cells incompatible for HLA class I molecules (Fig. 1). We could individualize two restricting elements for the NEF antigen: HLA-B8 in patient AB and HLA-A1 in patient BR. Similarly, the ENV-specific lysis observed in patient AB appeared to be MHC restricted, inasmuch as no lysis could be detected on ENV-expressing target cells that were totally mismatched for HLA class I or class II molecules. In contrast, although we could not esti-

mate the MHC class II restriction of patient BR whose HLA class II haplotype was not available, the primary response for ENV was not restricted by MHC class I molecules (Fig. 1 b). This finding is in agreement with previously described findings for ENV-specific cytotoxicity in peripheral blood (7). Cytotoxic responses specific for HIV were also analyzed in lymph node lymphocytes from two patients with PGL. Pathologic examination of lymph node sections revealed a follicular hyperplasia. In both cases, the NEF-specific CTL were detectable only after an in vitro restimulation, as shown in Fig. 2 for patient GO. This is in contrast to a low ENV-specific CTL activity which was detectable in primary response (Fig. 2 a).

These results were compared with data obtained in lungs from patients with HIV-related lymphocytic alveolitis. We had previously reported the detection of alveolar HIV-specific CTL in such patients by using as target cells P815 cells doubly transfected with HLA and HIV genes (10). As shown in Fig. 3 a, a



**Figure 2.** HIV-specific cytotoxic responses from lymph node cells of patient GO. (a) Primary CTL responses: lymph node lymphocytes (HLA A2-A19.2/B7-B<sup>-</sup>/DR7-DR10-DR53) were tested against a B cell line (HLA-A2-A3/B7-B40/DR4-DR8) infected by VAC-ENV (■), VAC-GAG (Δ), VAC-VIF (▲), VAC-TAT (◇), VAC-WT (●). (b) Secondary CTL responses from the same donor were tested after a 15-d stimulation with irradiated autologous cells and IL-2, against the same B cell line infected with VAC-ENV (▲), VAC-NEF (■), VAC-WT (●). (c) HLA restriction of secondary ENV-specific cytotoxic activity. In vitro stimulated effector cells were tested against HLA-A2/B7-matched (A2-A3/B7-B40/DR4-DR8) VAC-ENV (▲) and VAC-WT (○), HLA-B7-matched (A1-A3/B7-B<sup>-</sup>/DR13-DR4) VAC-ENV (■) and VAC-WT (□), or HLA-mismatched (A1-A25/B8-B18/DR3-W6) VAC-ENV (●).



**Figure 3.** HIV-specific primary cytotoxic responses of alveolar lymphocytes. (A) Alveolar lymphocytes from 12 patients were tested against HLA-matched target cells infected with ENV, GAG, and NEF RVV. Results from the 50/1 E/T ratio are given after subtraction of the control lysis obtained with WT virus. Lysis were considered as positive when above 5% of the mean control lysis ( $> 3$  SD). (B) Cytotoxic responses of patient 1 effector cells (HLA A1-A2/B8-B40/DR4-DR5-DR52-DR53) against EBV cell line (HLA-A2-A3/B7-B40/DR4-DR8) infected with VAC-ENV (■), VAC-GAG (●), VAC-NEF (▲), VAC-TAT (△), VAC-VIF (○), and VAC-WT (□). (C) HLA restriction of patient 1 alveolar effector cells was tested against B cell lines infected with VAC-ENV and mismatched for HLA-class I (A9-A<sup>-</sup>/B22-B35/DR4-DR5) (○) or for matched HLA-B8 (△) (A26-A26/B8-B8/DR2) or for HLA-A2/B40 (A2-A3/B7-B40/DR4-DR8) (■).

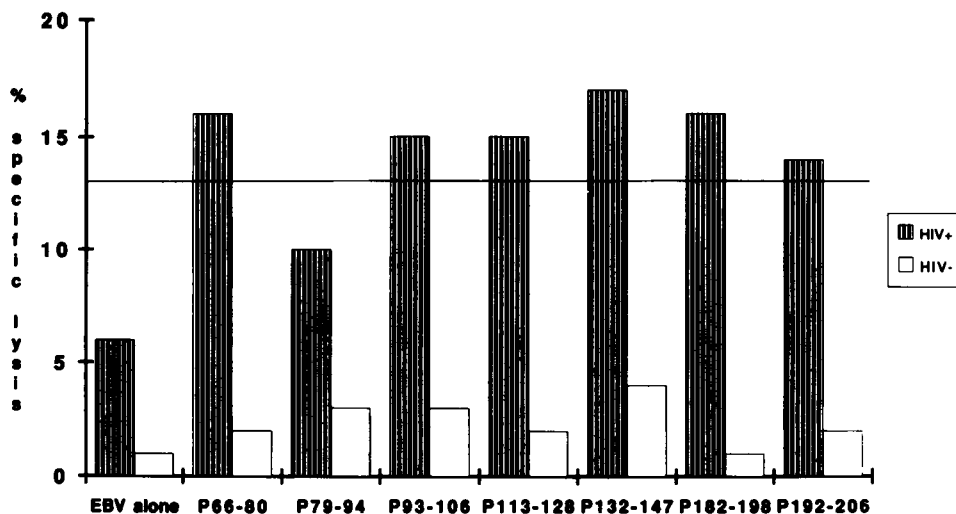
primary NEF-specific cytotoxic activity was detected in 4 of 12 patients, whereas it was detected in 3 of 6 patients for both ENV and GAG. We did not detect any lysis against TAT or VIF proteins (data not shown). These primary cytotoxic responses detected in lungs were restricted by HLA class I antigens for all three HIV proteins studied, including the ENV protein (Fig. 3 c).

Altogether, these data demonstrate that primary CTL responses specific for NEF are detectable in spleens as well as in lungs from seropositive patients with ITP or IP. Moreover, NEF is a target antigen for CTL similar to GAG or ENV HIV-1 proteins.

*Relative frequencies of CTL specific for immunodominant regions of the NEF protein.* The strong immunogenicity of NEF for CTL prompted us to identify the immunodominant epitopes recognized by CTL specific for HIV-1 NEF. We used a series of 17 partially overlapping peptides (12–17 AA in length) synthesized according to the HIV-BRU sequence. A preliminary screening of NEF epitopes recognized by splenic CTL was performed in bulk assays against an HLA-A1 compatible B-cell line coated with the corresponding peptides. The results are shown in Fig. 4. 11 peptides did not induce specific lysis as shown for the representative P79–94 peptide.

Among the 17 peptides tested, 6 were recognized by splenic CTLs. Strikingly, two regions were simultaneously recognized: a central 66–147 region (defined by peptides P66–80, P93–106, P113–128, P132–147) and an additional carboxyl-terminal 182–206 region (P182–198, P192–206). No specific cytotoxic activities were detected against these peptides in two HLA-A1 seronegative controls. The nature of primary effector cells specific for NEF was confirmed in depletion experiments with CD8-coated immunomagnetic beads and inhibition assays with anti-CD8 MAbs, indicating that the NEF-specific cytotoxic activities are mediated by CD8<sup>+</sup> lymphocytes (data not shown).

In order to further investigate these peptide-specific CTL, we quantified the relative frequencies of patient BR splenic cytotoxic effector cells directed against the two regions defined above. Results of LDA indicate high frequencies for effector CTL directed against the carboxyl-terminus peptides ( $5 \times 10^{-6}$  cells and  $2 \times 10^{-5}$  cells for P182–198 and P192–206, respectively). CTL specific for the central region of NEF had frequencies ranging between  $1.5 \times 10^{-4}$  and  $2 \times 10^{-6}$  (Fig. 5). In contrast, the LDA failed to detect any effector cells ( $< 10^{-8}$ ) reacting with peptides (P4–18, P46–60) that were not recognized in bulk assays (data not shown) or with the control B cell line alone.



**Figure 4.** Recognition of NEF peptides by splenic CTL. Splenic T cells from patient BR (HLA A1-A19/B44-B<sup>-</sup>) (■) and from a seronegative donor (HLA A1-A<sup>-</sup>/B8-B44) (□) were used as effectors at an E/T ratio of 80:1 in a <sup>51</sup>Cr release assay against B target cells (HLA A1-A25/B8-B18/DR3-W6/CW7) alone or preincubated with NEF peptides. The cutoff reflect the mean of lysis obtained with the control EBV cell line alone + 3 SD.

Lymphocytes per well

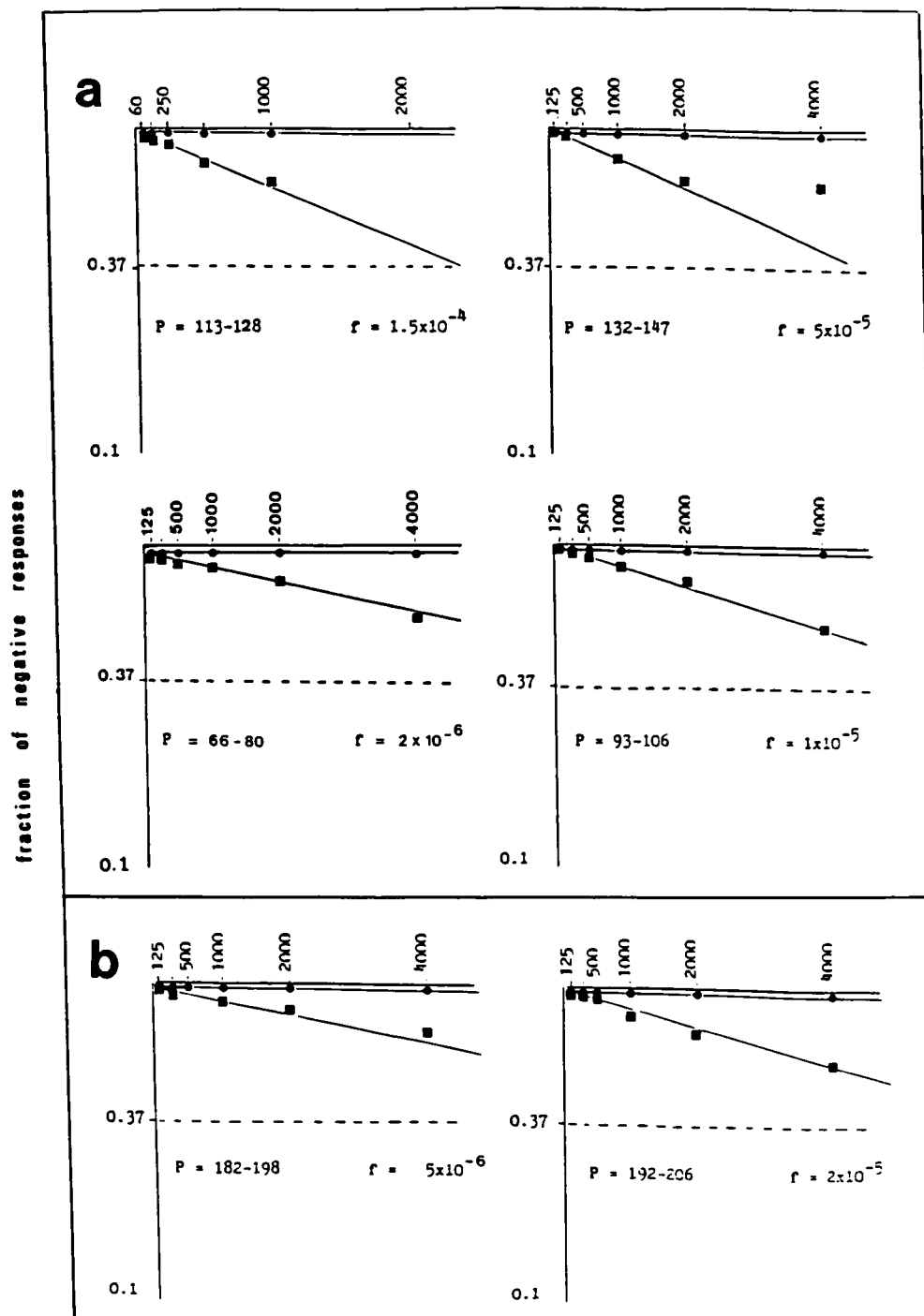


Figure 5. LDA of relative frequencies of splenic cytotoxic effector cells (HLA-A1-A19/B44-B<sup>-</sup>) directed against peptides localized within the (a) NEF central region (66-147), and (b) carboxyl-terminal part (182-206) using an HLA-A1-matched B cell line (A1-A25/B8-B18/DR3-W6/CW7) alone (●), or preincubated with defined peptides (■).

Thus we could define a new immunodominant region AA182-206 located in the carboxyl-terminal domain of NEF. Moreover, the LDA allowed us to determine the relative frequencies of *in vivo* differentiated splenic CTL specific for distinct HIV-1 NEF peptides.

*Presentation of the central and carboxyl-terminal region of NEF by HLA-A1 and -B8 molecules.* The above data suggest that HLA-A1-restricted CTL can recognize two distinct regions in the NEF protein. The MHC class I restriction of CTL responses specific for these regions were demonstrated, using P113-128 peptide (central domain) and P192-206 peptide (carboxyl-terminal domain) as examples (Fig. 6).

In order to confirm the ability of the HLA-A1 molecule to present the epitopes defined above, we performed similar LDA in several HLA-A1 patients. We selected alveolar lymphocytes from two HLA-A1 patients with CD8<sup>+</sup> lymphocytic alveolitis and studied their effector cell frequencies against the HLA-A1-matched B-cell line coated with P113-128 peptide. Interestingly, we obtained the same range of frequencies ( $1.5-3 \times 10^{-4}$ ) as observed for patient BR splenic lymphocytes (data not shown). The P113-128 peptide of the NEF molecule can thus be recognized in the context of the HLA-A1 molecule by splenic and alveolar lymphocytes from three distinct HIV-infected patients.

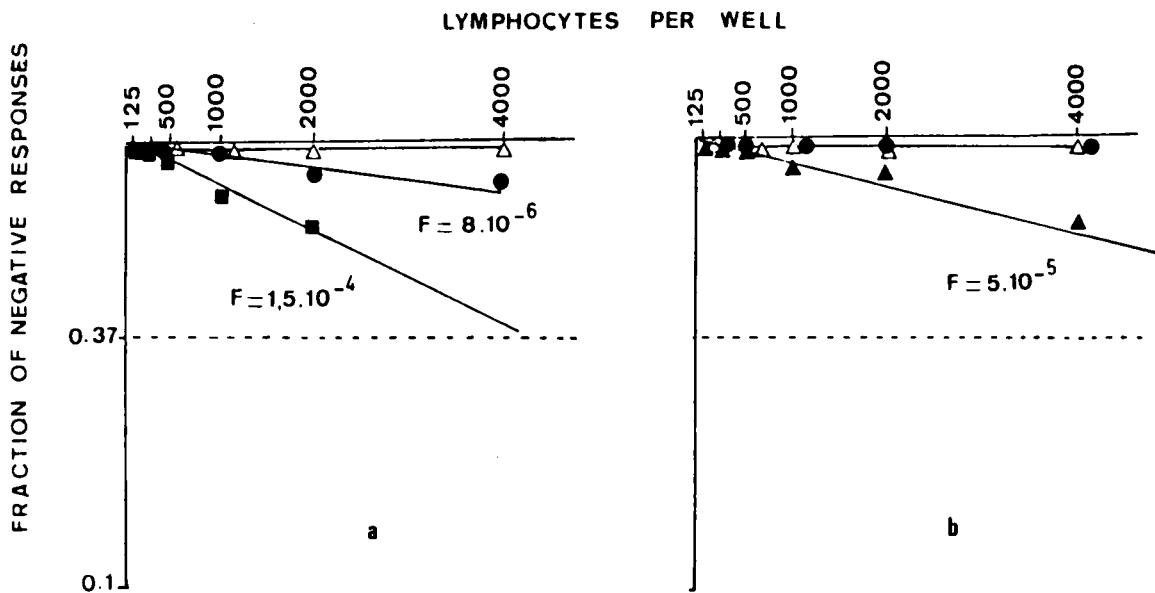


Figure 6. HLA-A1 restriction of peptide specific splenic effector cells. Patient BR (HLA A1-A19/B44-B<sup>-</sup>) effector cells frequencies were estimated by LDA in a primary cytotoxic assay. (a) Target cells are an HLA-A1-matched ( $\Delta$  A1-A25/B8-B18/DR3-W6/CW7) B cell line, alone ( $\Delta$ ) or an preincubated with P113-128 ( $\blacksquare$ ) and HLA-mismatched (A2-A3/B7-B40/DR4-DR8) B cell line ( $\bullet$ ). (b) Target cells are HLA-A1-matched ( $\Delta$  A1-A25/B8-B18/DR3-W6/CW7) B cell line, alone ( $\Delta$ ) or preincubated with P192-206 ( $\blacktriangle$ ) and HLA-mismatched (A2-A3/B7-B40/DR4-DR8) B cell line ( $\bullet$ ).

In contrast, when testing splenic lymphocytes from a fourth HLA-A1 patient AB, no HLA-A1-restricted primary cytotoxic effector cells could recognize either the NEF central P113-128 or the NEF carboxyl-terminus P182-198, P192-206 peptides (Fig. 7). Moreover, precursors of CTL specific for those peptides were not detectable in LDA after an *in vitro* restimulation of patient AB splenic lymphocytes with the HLA-A1 B-cell line coated with peptides 113-128, 182-198, or 192-206 (data not shown). Since the NEF-specific splenic CTL from this patient were restricted by the HLA-B8 rather than by the HLA-A1 antigen, we tested whether these peptides could be presented by the HLA-B8 molecule. Indeed, we could demonstrate that P182-198, but not P113-128 nor P192-206, was recognized in association with the HLA-B8 antigen, with an effector cells frequency of  $1 \times 10^{-4}$  (Fig. 7).

These overall data indicate that the HLA-A1 and B8 antigens are able to present efficiently epitopes located in both the central and carboxyl-terminal regions of NEF to CTL specific for the NEF protein.

## Discussion

In this study, we investigated cytotoxic T cell responses specific for HIV in lymphoid organs where both an active HIV replication and a massive CD8 infiltration have been observed (11-13). We report the first demonstration of primary specific cytotoxic T cell responses against HIV proteins detected in spleens from seropositive patients with ITP. These responses are similar to that observed in lungs from patients with HIV-related interstitial pneumonitis by our group (8-10) or in peripheral blood, by others (4-7). These observations question the role of tissular CTL either in the control or the pathogenesis of HIV infection. Previous reports (28, 29) demonstrated an active role for CD8<sup>+</sup> cells in the *in vitro* control of HIV replication. Similarly, the P24 antigen production in cocultures generated from patient BR CD8-depleted spleen cells was enhanced as com-

pared with cultures derived from total spleen cells (F. Hadida, unpublished data). Alternatively, tissular HIV-specific CTL could also be deleterious for the function of infected organs as demonstrated previously in the lung model by our group (8, 10, 30). Thus, lymphoid organs may represent a major reservoir for HIV-specific CTL which could participate both in the control of HIV replication level and in the subsequent follicle lysis.

The CTL responses directed against early proteins have been shown to play an active role in the control of viral replication or spread, in several viral infections (3, 20). Since NEF is an early regulatory protein which is not expressed in infectious particles, the NEF-specific CTL response could play a critical role in host cytotoxic defenses against infected cells at an early step of the HIV replication. The detection of NEF-specific CTL responses observed in lymphoid organs and lungs was similar to that observed against the structural ENV and GAG proteins, confirming the high immunogenicity for this regulatory protein (15-17). These data prompted us to further investigate the fine specificity of tissular CTL directed against NEF.

We report here a new antigenic region (AA182-206) located at the carboxyl-terminal region of the NEF molecule which is recognized by CTL in the context of HLA-A1 or B8 molecules, together with a set of peptides mapping in the previously published region located within AA73-82 and 113-128 (15, 17). Moreover, we report that two common HLA molecules, HLA-A1 and B8, are also able to present the P66-80, P93-106, P113-128, and P132-147 central peptides apart from the previously reported HLA A3, A11, B17, B37, B18 molecules (15, 17).

The observation that several HIV peptides can be simultaneously recognized by CTLs from a single donor seems rather unusual as compared with previous studies performed in other viral models where a single immunodominant epitope is usually recognized in association with a single HLA molecule (31, 32). However, those latter studies were performed on T cell

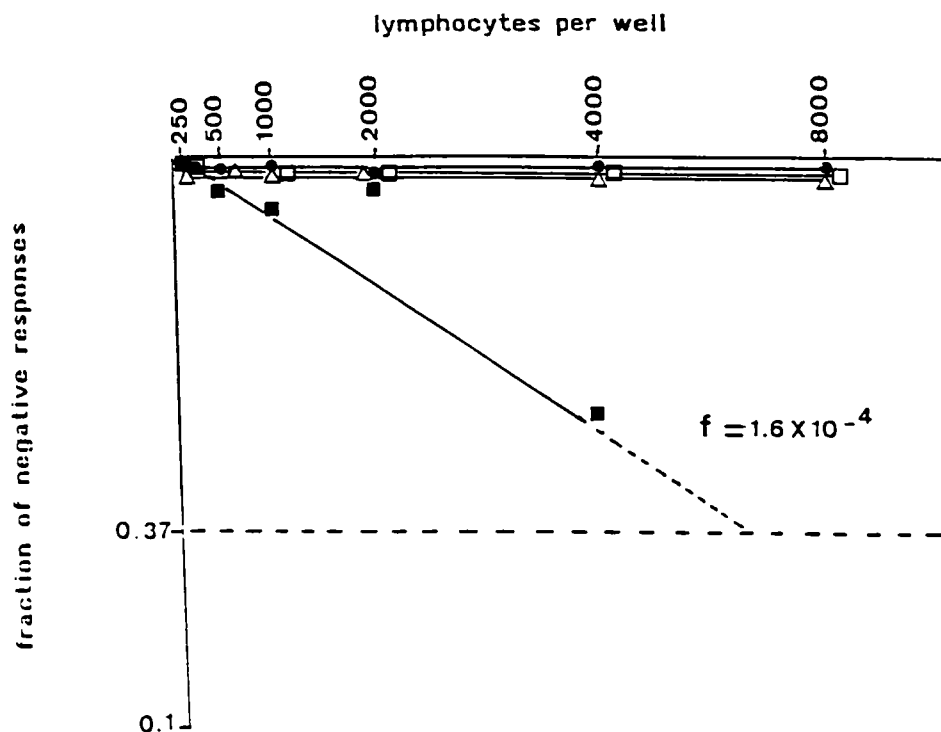


Figure 7. HLA-B8 restriction of splenic primary effector cells from patient AB (HLA A1-A29/B8-B12/DR1-DR7-DR53). Target cells are: B cell line (HLA A26-A26/B8-B8/DR2-DR2) preincubated with P183-199 (■) and P192-206 (□); B cell line (HLA A1-A25/B8-B18/DR3-W6/CW7) preincubated with P113-128 (●), or B cell line alone (Δ).

lines where a clonal expansion might be responsible for selection of a single immunodominant epitope. Conversely, mapping T cell epitopes in primary cytolytic assays requires that the specific differentiated CTL are present with high frequencies. The LDA allowed us to define the relative frequencies ( $1 \times 10^{-6}$  to  $1 \times 10^{-4}$ ) of epitope-specific primary CTL from a single donor that recognize six distinct peptides of NEF presented by the HLA-A1 molecule. Moreover, similar high frequencies were detectable both in lymphoid organs and lungs from several individuals, suggesting a lack of compartmentalization for HIV-specific CTLs. Thus the LDA seems a powerful tool to analyze epitopes recognized in primary CTL responses by avoiding the bias of clonal selection associated with the use of T cell lines. The unusual chronic antigenic stimulation, owing to the persistence of HIV replication in vivo, may play a critical role both in the differentiation of CTL precursors specific for a wide spectrum of NEF epitopes, and in the induction of such high CTL frequencies.

Interestingly, the comparison of our experimental definition of CTL epitopes in the NEF protein with three predictive models for T cell epitope localization, such as developed by Rothbard and Taylor (33), Margalit et al. (34), and Kourilsky and Claverie (35), allowed us to observe a striking parallel between these epitopes. Indeed, as shown in Fig. 8 and reviewed in Bahraoui et al. (21), at least two T cell epitopes map in the new region reported here between AA 182 and 206 of the NEF carboxyl-terminal region, and can be predicted within AA 188-196 and 198-204, from models defined above. Similarly, the experimentally defined CTL epitopes in the central (AA82-148) region contain several sequences predicted as T cell epitopes from the theoretical models. Several epitopes can also be predicted within regions defined by AA26-46, 152-160, and 172-184 that have remained silent up to now. Thus both the central and carboxyl-terminal regions of NEF could represent a continuum of CTL epitopes able to be presented in the context of several HLA molecules. These structural properties of NEF might explain its high immunogenicity. Finally, the

carboxyl-terminal region of the NEF protein described here for the first time as an immunodominant region for CTLs, appears to be particularly interesting in view of vaccine development strategies. Indeed it has also been reported as an immunodominant region for human and simian antibodies and T helper cells responses specific for NEF (21). In addition, this carboxyl-terminal region seems rather conserved among the several HIV strains and a consensus epitope can be defined between AA194 and 206 (G. Jung, personal communication).

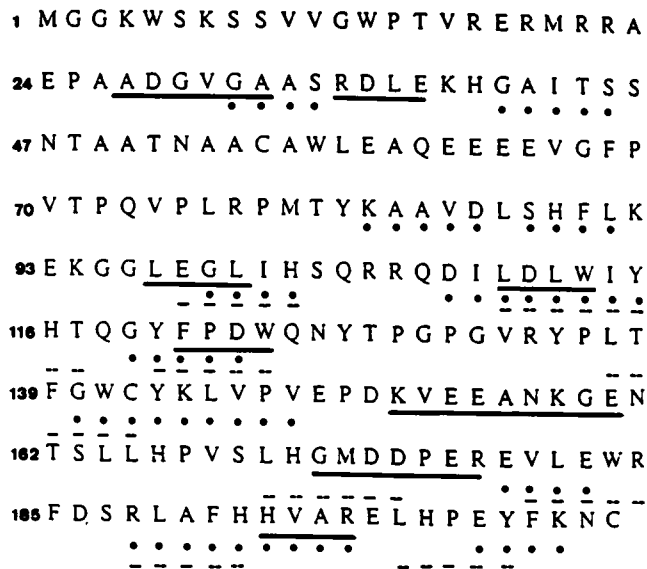


Figure 8. Comparison between three predictive models for T cell epitopes localization and experimental findings. Prediction according to the sequences (· · ·) of Rothbard and Taylor (33), or based on the rarity of tetrapeptide stretches (---), of Kourilsky and Claverie (35), or on amphipatic  $\alpha$ -helix according to the hydrophobicity scales (—) of Margalit et al. (34).

We are thus currently delineating the 182–206 carboxyl-terminal epitopes of NEF implicated in T cell recognition. One of our main goals will be to evaluate the protective efficiency of CTL specific for this carboxyl-terminal domain of HIV-1 NEF for therapeutic or preventive strategies.

## Acknowledgments

We thank M.-R. Marescot and P. Barban for HLA typing, Y. Dudoit for preparation of the EBV-transformed B cell lines, and Marie Korner for helpful discussion of the manuscript.

This work was supported by grants from Agence Nationale de Recherche sur le SIDA and Ministère de la Recherche et des Technologies.

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