

Impaired Cytotoxic T Lymphocyte Recognition Due to Genetic Variations in the Main Immunogenic Region of the Human Immunodeficiency Virus 1 NEF Protein

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Summary

Human immunodeficiency virus (HIV) induces strong responses from human histocompatibility leukocyte antigen (HLA) class I-restricted cytotoxic T lymphocytes (CTL). In a previous report we identified an immunodominant region (amino acids 73-144) in the NEF protein that was recognized by CD8⁺ class I-restricted CTL of most asymptomatic individuals. Analysis of the 73-144 region by peptide sensitization experiments using overlapping peptides corresponding to the LAI isolate identified the peptide sequences located between residues 73 and 82 or 84 and 92 and the peptide sequence between residues 134 and 144 as cognate peptides for HLA-A11- and HLA-B18-restricted epitopes, respectively. This report describes the variable demonstrable reactivities of CTL obtained from HLA-A11 or HLA-B18 seropositive, asymptomatic patients who all had a response to the virus NEF protein, but who did not always recognize appropriate cognate peptides. The high mutation rate of HIV probably facilitates the selection of mutants that can avoid the cellular immune response. We therefore analyzed the variability of these epitopes restricted by HLA-A11 and HLA-B18. We sequenced several viral isolates from HLA-A11 and HLA-B18 donors who recognized certain HLA-peptide complexes and from those who did not. A CTL sensitization assay was used to show that some mutations led to a great reduction in CTL activity *in vitro*. This might be due to failure of the mutated epitope to bind major histocompatibility complex class I molecule. A simple assay was used to detect peptides that promoted the assembly of class I molecules. Some of these mutations at major anchor positions prevented HLA-A11/peptide binding, and consequently impaired recognition of the HLA-peptide complex by the T cell receptor.

Individuals infected with HIV-1 generate a great diversity of CTL. These CTL are directed against several structural and regulatory HIV-1 proteins (1) and each protein can have multiple T cell epitopes (2-5). The rapid elimination of infected cells by CTL directed against proteins synthesized early in infection is an important defense against viral infection. CTL directed against the early-expressed NEF protein of HIV could provide protection *in vivo* by eliminating virus-infected cells before there is substantial release of virus particles. We have previously identified an immunodominant region in the central portion of the NEF protein (residues 73-144) that is recognized by most of the patients producing a CTL response to NEF in association with several HLA class I molecules (2, 3). This result pointed out the widespread recognition of conserved epitopes in the NEF protein. We used a peptide

sensitization assay to identify and describe three peptide sequences within this region; two are cognate peptides for HLA-A11-restricted epitopes (residues 73-82[QVPLRPMTYK] and residues 84-92[AVDLSHFLK]), and the third for HLA-B18 (residues 134-144[RYPLTFGWQYK]) (Culmann-Penciolelli, B., S. Lamhamedi-Cherradi, I. Coullin, N. Guegan, J. P. Levy, J. G. Guillet, and E. Gomard, manuscript submitted for publication). We have now obtained CTL from a geographically-defined cohort of HLA-A11 and HLA-B18 seropositive asymptomatic patients and tested their reactivities to these peptides. The CTL from certain of these patients did not recognize the appropriate cognate peptide(s). The high mutation rate of HIV could play a major part in selecting mutants having hidden functional epitopes, as recently described for GAG and ENV proteins of HIV (6-8).

We have therefore investigated these subjects to see if the lack of a CTL response to the NEF protein epitopes is due to viral mutation.

Materials and Methods

Lymphocytes. PBMC from asymptomatic HIV-1 seropositive donors were isolated in a density gradient and frozen in liquid nitrogen. HLA serotyping was performed by the tissue-typing laboratory of the Hôpital Saint-Louis (Paris, France): W8 [HLA-A11/26, B7/37, C6/7]; W13 [HLA-A25/32, B18/-, C8/-]; W19 [HLA-A2/19.2, B18/62, C5/-]; W24 [HLA-A2/11, B18/-, C7/-]; and W44 [HLA-A2/-, B18/44, C5/-].

Cytotoxic T Cell Assays. Polyclonal anti-HIV cell lines were established in culture for 2–3 wk using PBMC (10^6 /ml) with autologous PHA-activated lymphocytes (2×10^5 /ml) as previously described (3). CTL activity was assayed by a chromium release test (CRT), using EBV-transformed lymphoblastoid cell lines as target cells. They were prepared as previously described (2), either by infection with wild-type vaccinia virus VAC-WT or HIV-1/LAI *nef* recombinant vaccinia virus VAC-NEF (Transgène, Strasbourg, France), or by incubation with synthetic peptides corresponding to the HIV-1/LAI NEF sequence or to mutated sequences (Neosystem, Strasbourg, France). For peptide titration experiments, target cells were incubated with 10-fold serial dilutions of mutated or LAI reference peptides for 1 h (final concentrations: 0.003–3 μ g/ml).

Preparation of DNA. PBMC pellets were suspended in an equal volume of buffer (50 mM Tris, pH 8, 500 mM NaCl, 20 mM EDTA), and the cells were lysed by adding one volume of buffer containing 1% SDS. The lysates were treated with RNase A (0.1 mg/ml, 30 min at 37°C) and proteinase K (0.4 mg/ml, overnight at 37°C), and DNA isolated by phenol/chloroform extraction and ethanol precipitation.

PCR, Cloning, and Sequencing of NEF Protein. The PCR reactions were carried out in a 100- μ l reaction mixture containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM MgCl₂, 0.2 mM each dNTP, 0.4 μ g of each primer, 1.5 U *Taq* polymerase (Perkin Elmer Cetus, Norwalk, CT), and 2 μ g PBMC cell DNA. The primers were NEF5S 5'GTGTCGACAAGAAT(N)AGACAGGGCTT(R)GAAAG-3' (sense; SaI site underlined) and NEF3R 5'ACGAATTC TGGAAAGTCCCCAGCGGAAAGT-3' (antisense, EcoRI site underlined) corresponding to nucleotides 8352–8375 and 9031–9052 of the HIV-1/LAI sequence, respectively. Samples underwent 40 amplification cycles. Each cycle consisted of three steps: denaturation at 92°C for 1.5 min, annealing of primers at 58°C for 1.5

min, and extension at 72°C for 1.5 min. In the first cycle, sample were denatured at 94°C for 4 min, and in the last cycle, the extension step was at 72°C for 5 min. Amplified products were digested with restriction enzymes (*SaI* and *EcoRI*) purified, and cloned in a M13mp18 vector digested by *SaI* and *EcoRI*. For each patient, the whole *nef* gene for different clones was sequenced (model 373A sequencer; Applied Biosystems, Inc., Foster City, CA).

Detection of HLA-A11 Assembly by ELISA. The assay (9) used T2 cells expressing HLA-A11 molecules (10) (a gift of Dr. M. G. Masucci, Karolinska Institute, Stockholm, Sweden). Briefly, aliquots of 0.8×10^6 T2-A11 cells were lysed in 64 μ l 10 mM Tris-buffered saline, pH 7.5, containing 1% NP-40, protease inhibitors, and 1 μ M exogenous peptide. The mixture was incubated in microfuge tubes (model 0030102.002; Eppendorf, Hamburg, Germany) at 37°C for 30 min, and HLA-A11 assembly was detected on microtiter plates coated with Ig A11.1M (11) (1 μ g in 100 μ l PBS per well). The equivalent of 4×10^5 cells in 100 μ l medium per well was incubated with Ig for 20 h at 4°C. It was then incubated with the second Ab, an anti- β 2 microglobulin, M28 Ig (12), labeled with biotin (2 μ g/ml) for 1 h at 37°C. Reaction products were detected by incubation with ExtrAvidin-alkaline phosphatase (E-2636; Sigma Chemical Co., St. Louis, MO) for 45 min at 22°C. Alkaline phosphatase activity was detected with the *p*-nitrophenyl phosphate disodium hexahydrate as substrate (104-105; Sigma Chemical Co.) and the resulting absorbance was measured at 405–492 nm.

Results and Discussion

We have attempted to obtain further insight into the mechanisms by which persistent virus evades CTL. The nucleotide sequences of the *nef* genes of several HIV-1 clones from donors whose CTL recognized the cognate peptides for HLA-A11 or HLA-B18-restricted epitopes in the immunodominant region of NEF residues 73–144 (2–4) and from donors whose CTL did not recognize one or more of these peptides were determined. The deduced amino acid sequences were compared with that of the LAI isolate used to define the reference NEF epitopes.

As shown in Fig. 1, CTL from donors W8 and W24 recognized two distinct epitopes located in the central region of the NEF protein in association with the HLA-A11 molecule. Peptide 84–92 was recognized by donor W8, but not peptide 73–82, whereas the reverse occurred with donor W24.

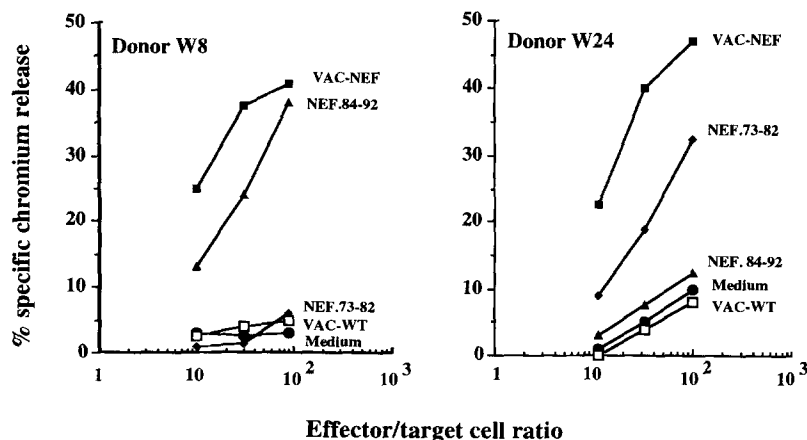


Figure 1. Cytotoxic activity of CTL prepared from the PBMC of W8 and W24 HLA-A11 donors against two HLA-A11-restricted epitopes in the central region of NEF protein. Effector cells were prepared as described in Materials and Methods. Target cells shared only the HLA-A11 molecule with effector cells, and were either infected with VAC-WT or VAC-NEF, or incubated with synthetic peptides NEF 73–82 and NEF 84–92.

DONOR	VIRAL CLONES	73	82	84	92	94
	LAI	T P Q V P L R P M T Y K	A	V D L S H F L K	E	K
W8	8-1	R	-	F - G - L	-	-
	8-2	K	-	- G - L	-	-
	8-3	K	-	- G - L	-	-
	8-4	K	-	- G - L	-	-
	8-5	K	-	- G - L	-	-
	8-6	K	-	- G - L	-	-
	8-7	K	-	- G - L	-	-
	8-8	K	-	- G - L	-	-
	8-9	K	-	- G - L	-	-
	LAI	T P	G V P L R P M T Y K	A A V D L S H F L K	E	K
W24	24-1	R	-	S R - R	-	-
	24-2	R	-	- F - R	-	R
	24-3	R	-	S R - R	-	-
	24-4	R	-	S R - R	-	-

Figure 2. Mutations in the putative anchorage residues (in italic and bold type) or in flanking residues of two HLA-A11-restricted NEF epitopes from different isolates from HIV-infected donors W8 and W24. The LAI peptides recognized by CTL from these donors (see Fig. 1) are shown in gray boxes.

The differences between these two donors are not related to subtypes of HLA-A11 molecules, as defined by cellular tests (13), since the B-EBV cells of donors who did not recognize one of the cognate peptides can present this peptide to CTL from donors whose CTL recognize the nonmutated epitope. All 13 virus isolates examined from donors W8 and W24 had mutations in position 2 (Val to Leu, Phe, or Arg) of peptide 84-92, and one isolate recovered from donor W24 also had a Lys for Arg substitution in position 9 (Fig. 2). It is more than likely that the Val in position 2 and Lys in position 9 are the anchoring residues for HLA-A11, since they are also found in other HLA-A11-restricted epitopes, such as peptide NEF 73-82 and peptide 416-424 of the nuclear antigen 4 of EBV (10). We tested to see whether the corresponding mutated peptides could sensitize target cells to CTL

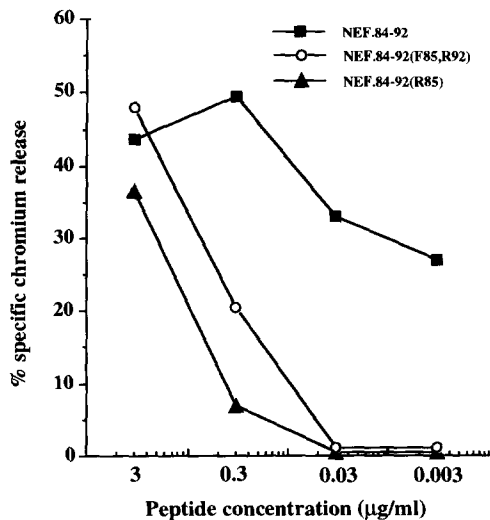


Figure 3. Titration of LAI reference peptides or mutated peptides corresponding to the HLA-A11-restricted 84-92 epitopic region. Effector cells were CTL generated from the PBMC of donor W8 and tested against autologous target cells. The E/T cell ratio was 230:1.

lysis. The Val to Leu change in position 2 of the W8 isolate did not influence recognition of the 84-92 epitope by HLA-A11-restricted CTL. In contrast, the other mutations in the W24 isolates all affected the peptide sensitization of target cells to lysis by CTL from donor W8 (Fig. 3). Peptide titration experiments demonstrated that cognate peptide caused sensitization at extremely low concentrations, whereas peptides containing the putative mutations in the anchoring residues (Val to Arg in position 2, or Val to Phe in position 2 together with a mutation Lys to Arg in position 9) were required in correspondingly higher concentrations. We also used a simple assay to detect peptides promoting the assembly of HLA class I molecule in T2-A11 cells in order to determine whether mutations in the epitopic region prevented binding of the corresponding peptide to HLA molecules or affected the direct recognition by the TCR. Synthetic peptides corresponding to these mutations did not promote HLA-A11 assembly (Fig. 4). These results are consistent with previous observations that a peptide of a nucleoprotein from influenza virus with an Arg in position 2 did not bind to HLA-A3, a molecule very similar to HLA-A11 in most of the regions responsible for peptide binding specificity (14), and a Lys to Thr substitution in position 9 impaired HLA-A11 binding of EBV-derived epitopes (15). These results therefore show that W24 isolates contain mutations in residues that are required for effective peptide binding.

The recognition of the 73-82 region by CTL from donor W24 (Fig. 1), is probably due to the presence of the wild-type sequence in the isolates from this donor (clone 24.2, Fig. 2), since substitutions corresponding to the mutations identified in the W24 isolates (Tyr to Ser in position 9 and Lys to Arg in position 10) resulted in a very low binding to HLA-A11 (Fig. 4).

Mutations in anchoring residues might not be the only mechanism by which mutants evade the HLA-A11-restricted CTL response. The regions corresponding to the NEF epitope 73-82 were entirely conserved in nine isolates from donor

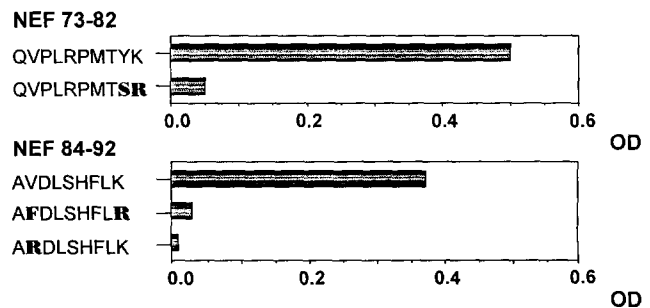


Figure 4. Assembly of HLA-A11 molecules in the presence of Nef peptides. Peptides (1 µM) were incubated in T2-A11 cells lysates as described in Materials and Methods. ELISA results are expressed as OD at 405/492 nm. The spontaneous assembly obtained with T2-A11 lysate without adding exogenous peptide was subtracted. It was 0.310 ± 0.025 (mean of several experiments) after incubation with alkaline phosphatase substrate for 1 h. The assembly ratio was 2.56 ± 0.20 for peptide Nef 73-82 and 2.19 ± 0.36 for peptide Nef 84-92. Mutated residues in the peptide sequences are indicated in bold type.

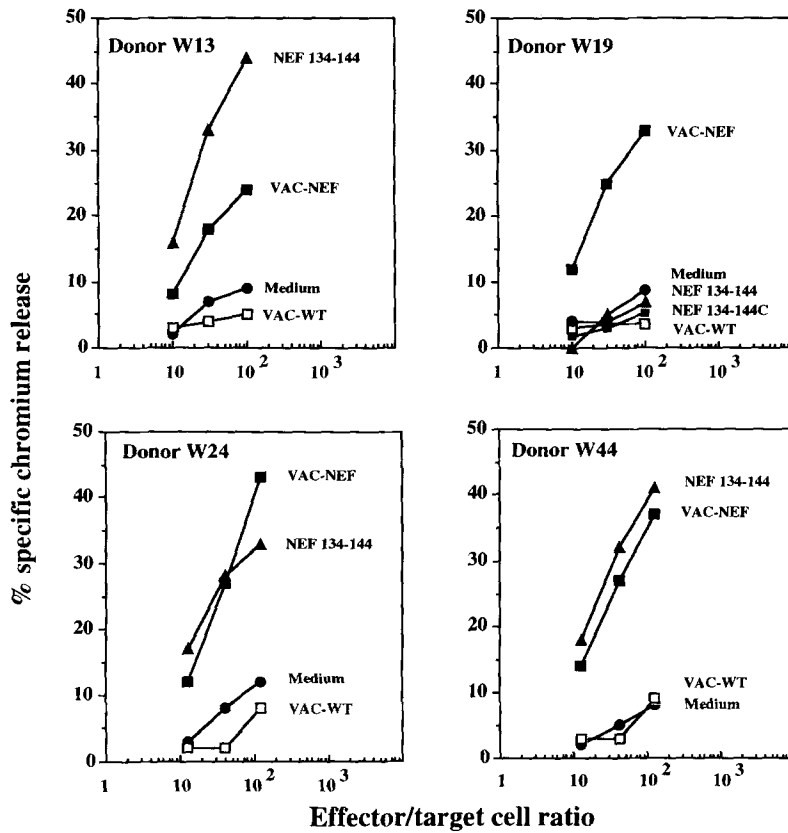


Figure 5. Cytotoxic activity of CTL prepared from donors W13, W19, W24, and W44 against HLA-B18-restricted NEF 134-144 peptide. Effector cells were prepared as described in Materials and Methods. Target cells shared only the HLA-B18 molecule with effector cells, and were either infected with VAC-WT or VAC-NEF, or incubated with NEF 134-144 synthetic peptides. NEF-specific CTL from donor W19 recognized another epitope, NEF 117-127, in association with HLA-B62 (data not shown).

W8 who did not recognize this epitope, whereas there were mutations in the flanking positions 71 (Thr to Arg or Lys) and 83 (Ala to Gly) (Fig. 2). This suggests that mutations in flanking regions may hinder antigen processing, as has been described for another viral system (16). Such mutation could affect the stoichiometry of a defined immunodominant epi-

tope, which may explain the presence of another HLA-A11-restricted epitope in these isolates.

The mechanism by which the mutants isolated from donor W19 evade HLA-B18-restricted CTL responses, whereas isolates from three other HLA-B18 donors do not (Fig. 5), is less clear. All three W19 isolates have a Thr to Cys mutation

DONORS	VIRAL CLONES	134	138	144								
W13	LAI	R	Y	P	L	T	F	G	W	C	Y	K
	13.1	-	-	-	-	-	-	-	-	-	F	-
	13.2	-	-	-	-	-	-	-	-	-	F	-
	13.3	-	-	-	-	-	-	-	-	-	F	-
	13.4	-	-	-	-	-	-	-	-	-	F	-
13.5	-	-	-	-	-	-	-	-	-	F	-	
W19	LAI	R	Y	P	L	T	F	G	W	C	Y	K
	19.1	-	-	-	-	C	-	-	-	-	F	-
	19.2	-	-	-	-	C	-	-	-	-	F	-
	19.3	-	-	-	-	C	-	-	-	-	F	-
W24	LAI	R	Y	P	L	T	F	G	W	C	Y	K
	24.1	-	F	-	-	-	-	-	-	-	F	-
	24.2	-	F	-	-	-	-	-	-	-	F	-
	24.3	-	F	-	-	-	-	-	-	-	F	-
24.4	-	-	-	-	Y	-	-	-	-	F	-	
W44	LAI	R	Y	P	L	T	F	G	W	C	Y	K
	44.1	-	-	-	-	-	-	-	-	-	F	-
	44.2	-	-	-	-	-	-	-	-	-	F	-
44.3	-	-	-	-	-	-	-	-	-	F	-	

Figure 6. Mutations in the HLA-B18-restricted NEF 134-144 epitope in isolates from four donors. The clones sequenced from each HIV-infected donor represent different isolates and are compared to the reference sequence of the LAI isolate. The LAI peptide recognized by CTL from each donor (see Fig. 5) is shown in gray boxes.

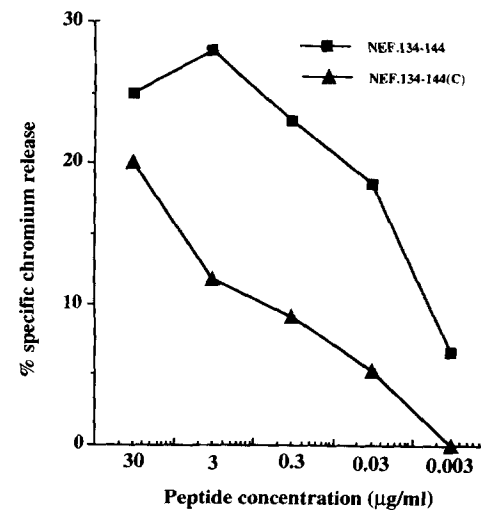


Figure 7. Titration of LAI reference peptide or mutated peptide corresponding to the HLA-B18-restricted NEF 134-144 epitopic region. Effector cells were obtained from donor W44, and were tested against HLA-B18 target cells (W19 B-EBV). The E/T cell ratio was 100:1.

in residue 138, in the region of the previously described NEF epitope 134-144 for HLA-B18 (Fig. 6). The corresponding mutated peptide did not sensitize target cells to lysis by CTL from W19 (Fig. 5). The CTL from donor W44, which responded to the standard HIV-1/LAI NEF peptide (amino acids 134-144) recognized this sequence even when it was presented on B-EBV cells from donor W19, that had the same restriction element. But they recognized the mutant peptide poorly unless very high peptide concentrations were used (Fig. 7). It is not yet possible to determine whether cysteine 138 induces a lower antigenicity for CTL, or weaker binding to the groove of HLA-B18 molecule, since the anchoring residues for HLA-B18 molecules have yet to be determined and T2-B18 cells are not available for binding experiments. We have also been unable to generate mutated peptide-induced CTL lines using W19 PBMC, although this approach was used

to determine nondominant epitopes (3, 17) which may be limited by the TCR repertoire.

These results show that the mechanism for generating CTL escape mutants is complex. Mutations were found that influence the avidity of the peptide for HLA molecules. Other mutations probably influence antigen processing, and/or recognition of the HLA-peptide complex by the TCR. Some mutations may also induce specific T cells to anergy (18, 19). These HIV mutants may persist in a population. Tests on antigenicity of the reference NEF/LAI isolate carried out in Paris between 1987 and 1993, show a marked decrease in its ability to induce recall CTL responses (results not shown). In summary, selection pressure may result in mutations in epitopes of the NEF protein that are important for efficient CTL recognition, and these may, in turn, facilitate virus persistence.

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