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.

Individually 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[RYPLTFGWCYK]) (Culmann-Penciolelli, B., S. Lamhamed-Cherradi, I. Couillin, 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-A1/26, 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 x 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 (Transgene, Strasbourg, France), or by incubation with synthetic peptides corresponding to the HIV-1/LAI NEF sequence or to mutated sequences (Neo-system, 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 ~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-~l reaction mixture containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM MgCl2, 0.2 mM each dNTP, 0.4 ~g of each primer, 1.5 U Taq polymerase (Perkin Elmer Cetus, Norwalk, CT), and 2 ~g PBMC cell DNA. The primers were NEF5S (GGTGTGACAAAGAT(N)AGACAGCGCTT(R)G AAGG-3′ (sense; SalI site underlined) and NEF3R 5′ACGAATTC TGGAAGTGCCCAGCGGAAATG-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 (SalI and EcoRI) purified, and cloned in a M13mp18 vector digested by SalI and EcoRI. For each patient, the whole nef gene for different clones was sequenced (model 373A sequencer; Applied Biosystems, Inc., Foster City, CA).

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.
Mutations in position 2 (Val to Leu, Phe, or Arg) of pept

All 13 virus isolates examined from donors W8 and W24

from donors whose CTL recognize the nonmutated epitope.

The differences between these two donors are not related to

antigen 4 of EBV (10). We tested to see whether the corre

sponding mutated peptides could sensitize target cells to CTL

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 pep

2-92, 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 posi

tion 9 are the anchoring residues for HLA-A11, since they

are found in other HLA-A11-restricted epitopes, such as pept

ide NEF 73-82 and peptide 416-424 of the nuclear

antineg 4 of EBV (10). We tested to see whether the corre

sponding mutated peptides could sensitize target cells to CTL

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 titra

tion experiments demonstrated that cognate peptide caused

sensitization at extremely low concentrations, whereas pep

tides containing the putative mutations in the anchoring

tides (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 deter

mine whether mutations in the epitopic region prevented

binding of the corresponding peptide to HLA molecules or

affected the direct recognition by the TCR. Synthetic pep

tides 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 there

fore 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

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 epi-

tope 73-82 were entirely conserved in nine isolates from donor

Figure 2. Mutations in the putative anchorage residues (in italic and

bold type) or in flanking residues of two HLA-A11-restricted NEF epi-
topes from different isolates from HIV-infected donors W8 and W24. The
LA1 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 pep

2-92, 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 posi

tion 9 are the anchoring residues for HLA-A11, since they

are found in other HLA-A11-restricted epitopes, such as pept

ide NEF 73-82 and peptide 416-424 of the nuclear

antineg 4 of EBV (10). We tested to see whether the corre

sponding mutated peptides could sensitize target cells to CTL

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 titra

tion experiments demonstrated that cognate peptide caused

sensitization at extremely low concentrations, whereas pep

tides containing the putative mutations in the anchoring

tides (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 deter

mine whether mutations in the epitopic region prevented

binding of the corresponding peptide to HLA molecules or

affected the direct recognition by the TCR. Synthetic pep

tides 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 there

fore 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 24.2A

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 epi-

tope 73-82 were entirely conserved in nine isolates from donor

Figure 3. Titration of LAI reference peptides or mutated peptides cor-

responding 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.

Figure 4. Assembly of HLA-A11 molecules in the presence of Nef pep-

tides (1 #M) were incubated in T2-All 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.
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 epitope, 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

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**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.

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**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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