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Identification of Multirestricted Immunodominant Regions Recognized by Cytolytic T Lymphocytes in the Human Immunodeficiency Virus Type 1 Nef Protein

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Peripheral blood mononuclear cells from a large number of human immunodeficiency virus (HIV)-seropositive donors were used to analyze the CD8$^+$ T-cell response to each part of the Nef protein of HIV-1/LAI. This report identifies an immunodominant region (amino acids 73 to 144) in the Nef protein that was recognized by 97% of the NEF responder donors. This peptide sequence was dissected into four epitopic regions (amino acids 73 to 82, 83 to 97, 113 to 128, and 126 to 144), each of which was recognized under different HLA class I restrictions. Short overlapping peptides were used to sensitize the target cells for cytolysis and so to determine if these epitopic regions were multirestricted. Each region was found to contain several epitopes recognized with different HLA molecules. Thus, the central region of the Nef protein, a regulatory protein expressed early in HIV-infected cells, is rich in epitopic sequences which are found to be similar in many infected individuals and which can be recognized in association with at least ten HLA class I molecules. Their implications for the vaccination of humans with peptide sequences are discussed.

Cytolytic T lymphocytes (CTL) are critical for recovery from many virus infections. Since recognition by antiviral CTL leads to lysis of virus-infected cells early in the replication cycle and prior to the release of infectious virus, we were interested in identifying the antigenic sequences in human immunodeficiency virus type 1 (HIV-1) regulatory proteins. As Nef is among the first and one of the major HIV-1 proteins produced following infection, we have investigated whether it is a recall antigen in HIV-seropositive individuals. We have previously shown that the majority (about 2/3) of HIV-seropositive donors generate Nef-specific CTL (16). These CTL precursors have also been found with great frequency in the peripheral blood of humans infected with other virus such as Epstein-Barr virus (EBV) (3), and their presence is associated with persistent activation of specific CTL. We have also used synthetic peptides to sensitize target cells in a functional assay (26) and defined several antigenic regions (amino acid sequences containing more than one T-cell epitope) in the central part of this protein that are recognized in association with different HLA class I molecules (5, 6). The present work extends our previous observations to show that at least 10 HLA class I molecules can be used as restriction elements by the CTL specific for the central part of the Nef protein. The study then examines whether the multiple recognition of a particular protein by major histocompatibility complex (MHC) class I molecules is due to cross-restriction or to multiple epitopes in this region. This type of cross-restriction has been described in other viral systems, including influenza A virus (1) and murine lymphocytic choriomeningitis virus (20).

Direct access to MHC-bound peptides recently became possible with the development of acid extraction methods (reviewed in reference 21). These studies demonstrate the presence of peptides in the groove of the MHC class I molecules and the possibility of using peptides eluted from these molecules to sensitize target cells. The results indicate that the minimal and optimal peptide length is 8 to 10 amino acids. We have attempted to determine how the Nef antigenic region adapts to the structural constraints imposed by haplotype-specific motifs in MHC restriction by using a functional assay to define more precisely the minimum epitopes recognized in the central region of the Nef protein by seropositive patients according to their HLA typing. These results indicate that these peptides may be suitable for use in the development of a human vaccination system.

MATERIALS AND METHODS

Lymphocyte donors. Heparinized blood samples were collected from 32 asymptomatic HIV-1-seropositive individuals being treated at two Paris hospitals, Hôpital Cochin (coded W) and Hôpital Pitié-Salpétrère (coded P). All individuals taking part in the 4-year study were in Centers for Disease Control disease stages II or III and had CD4 cell counts of 234 to 905/mm^3. Peripheral blood mononuclear cells (PBMC) were isolated in a density gradient (lymphocyte separation medium; Flow) and used directly after isolation or after being frozen in liquid nitrogen. HLA serotyping was performed by the tissue-typing laboratory of the Hôpital Saint-Louis (Paris, France).

Peptides. Peptides corresponding to the Nef sequence of HIV-1/LAI (18) were synthesized by Neosystem (Strasbourg, France) and supplied by the Agence Nationale de Recherche sur le Sida. Two sets of peptides were used in these experiments. The first included eight long overlapping peptides (corresponding to amino acid sequences 1 to 36, 34 to 71, 66 to 100, 93 to 125, 115 to 146, 137 to 168, 155 to 185, and 182 to 206). The second included 44 shorter peptides (overlapping peptides 8 to 16 amino acids long) corresponding to the central region of the Nef protein (sequence 73 to 147). Lyophilized

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peptides were dissolved in water (2 mg/ml) and stored at −20°C.

Generation of polyclonal anti-HIV cell lines. PBMC (10^6/ml) were cultured with autologous phytohemagglutinin-activated lymphocytes (2×10^5/ml) prepared as described by others (19) and irradiated (10,000 rads). The culture medium was RPMI 1640 supplemented with 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2 mM L-glutamine, nonessential amino acids, 1 mM sodium pyruvate, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, and 10% heat-inactivated human serum AB. After a 3- to 4-day incubation, cultures were maintained at a dilution of 10^6 cells per ml in culture medium supplemented with 5% interleukin 2-containing medium (12).

Generation of antipeptide cell lines. Primary cultures were obtained, in the same culture medium as described above, by coculture of 10^7 PBMC (5×10^6/ml) with the same number of autologous PBMC previously pulsed with 30 μg of peptide for 90 min and irradiated. Cells were cocultured for 7 to 9 days and then given a secondary in vitro stimulation by setting up 10^6 responding cells with the same number of stimulating cells in 1 ml of culture medium supplemented with 5% interleukin 2-containing medium. Thereafter, continuously growing cell lines were established by similar weekly stimulation. Only short peptides (8 to 15 amino acids long) were used as stimulating antigens.

Chromium release test. The target cells used in the chromium release test were lymphoblastoid cell lines obtained by transforming PBMC with EBV. They were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ (Dupont de Nemours) for 1 h and washed twice. Two protocols were used, depending on the length of the peptide. For smaller peptides (up to 15 amino acids long), 3×10^5 labelled cells were incubated with 10 μg of peptide in 1 ml of culture medium for 90 min, extensively washed, and then tested. For longer peptides (about 35 amino acids long), 5×10^5 cells were incubated (before being labelled) with 60 μg of each peptide in 1 ml of culture medium for 18 h, washed, and then labelled. The chromium release test was performed with microtiter plates by mixing of various concentrations of effector cells and 5×10^3 target cells in culture medium and incubation of them for 4 h at 37°C. The supernatants were then harvested, and the chromium released was measured in a gamma counter. Average spontaneous release values were 10 to 20% of the total Cr incorporated. The specific lysis was calculated as 100 × (experimental − spontaneous release)/(total Cr incorporated − spontaneous release).

RESULTS

Preferential recognition of the central region of the Nef protein by Nef-specific CTL. Previous study with four seropositive donors suggested that the Nef-specific CTL were preferentially elicited by the central region of this protein (5). The epitopic regions of the Nef protein involved in the recognition by CTL were determined by testing of HIV-specific T-cell lines from 32 seropositive donors capable of developing Nef-specific CTL against target cells expressing different parts of this protein. These target cells were pulsed with each of the eight long overlapping Nef peptides described in Materials and Methods. Table 1 shows that the CTL epitopes detected were preferentially located in three peptide regions, 66 to 100, 115 to 146, and 182 to 206; these were recognized by 69, 63, and 50% of Nef responder donors, respectively. There was no correlation between the preferentially recognized Nef region and the CD4$^+$ cell counts of these donors. All but one Nef responder donor (97%) recognized the 66-to-146 sequence, indicating that the central region of the protein was immunodominant for the Nef-specific CTL response.

Presence of four epitopic regions in the central part of the Nef protein. As the recognition of the central region of the Nef protein was recognized by most responder donors, the epitopes recognized by these CTL were more precisely located by use of a panel of short peptides (10 to 16 residues). The effector cells were derived mainly from polyclonal cell lines. Antipeptide cell lines were established to amplify weak activities against some peptides, and recognition of the corresponding endogenous epitope was checked with target cells infected with vaccinia virus containing the *nef* gene, as previously described (5). The presenting HLA molecules were tested by comparison of the lysis of autologous and partially HLA-compatible target cells in the presence of the corresponding peptide. Each HLA-A, -B, or -C of the donor was tested with several target cells. Only HLA-A and -B molecules were involved in the presentation of the Nef peptides to CTL, as illustrated with some of these target cells in Fig. 1 and further elucidated in Table 2. This experimental approach allowed us to define four epitopic regions in the central part of the Nef protein (73 to 82, 83 to 97, 115 to 128, and 126 to 144) (Fig. 1). The overlapping epitopes contained in each of these regions and recognized in association with different HLA class I molecules are described below.

CTL epitopes in region 73 to 82. The 73-to-82 region was recognized by Nef-specific CTL from six donors in association with three HLA molecules: donor W24 with HLA-A11, donors W30 and W47 with HLA-A3, and donors W33, W36, and P6 with HLA-B35. Each HLA restriction is illustrated for one donor in Fig. 1A to C. The use of five truncated peptides showed that two epitopes could be identified, even in this short sequence of 10 amino acids (Table 3). W24 and W47 CTL recognized only peptide 73 to 82, whereas W33 CTL also recognized peptide 74 to 81 with a similar lysis level. Thus, amino acids 73 and 82 are not essential to the epitopic peptide associated with HLA-B35, but they are involved with HLA-A3- and -A11-restricted epitopes. Therefore, the optimum peptide encompassing this antigenic region will differ with each particular HLA region.

CTL epitopes in region 83 to 97. The 15-residue region 83 to 97 was not only recognized in association with HLA-A11 by donors W8 and W24, as previously described (5), but also with HLA-B62 by donors W19 and P1 and with HLA-B8 by donors P1 and P10, as illustrated in Fig. 1D to F. This region contained at least three epitopes, at residues 84 to 92, 84 to 91, and 90 to 97, presented by HLA-A11, HLA-B62, and HLA-B8, respectively (Table 4). W8 CTL recognized the 84-to-92 nonapeptide but not 86 to 94, indicating that amino acids 84 and/or 85 are involved in the epitope recognized in association with HLA-A11. This 84-to-92 peptide could sensitize target cells at least at 0.003 μg/ml, suggesting that it corresponds to the exact epitope recognized with the HLA-A11 molecule (data not shown). On the other hand, P10 CTL lysed only target cells sensitized with peptide 90 to 97. It is all the more probable that the epitope recognized with HLA-B8 corresponded to this octapeptide, because it is still recognized by P10 CTL at a final dilution of 10^{-5} μg/ml (i.e., about 10^{-11} M), whereas peptides 86 to 97 and 90 to 100 were recognized only up to dilutions of 10^{-3} and 10^{-1} μg/ml, respectively (Fig. 2). The same results were obtained with P1 CTL when tested on HLA-B8-compatible target cells. This donor, P1, also generated another set of CTL in vitro that recognized peptides 83 to 94, 84 to 94, 83 to 91, and 84 to 91 on autologous and HLA-B62-compatible target cells, but not on HLA-B8 target cells. This suggests that the epitope associated with HLA-B62 corresponds to the

TABLE 1. Regions of the NEF protein recognized by specific CTL

Donor[a]	Specific Cr release with indicated target peptide[b]							
	1–36	34–71	66–100	93–120	115–146	137–168	155–185	182–206
W2	−	−	++	−	+++	−	−	++
W8	−	−	++	−	+++	−	−	−
W13	−	−	−	−	++	−	−	−
W14	−	−	−	−	−	−	−	++
W16	−	−	+++	++	+	−	−	−
W19	−	−	++	−	++	−	−	+++
W20	−	−	−	−	++	−	−	−
W21	−	−	−	−	++	−	−	+
W22	−	−	++	−	+++	−	−	−
W24	−	−	++	−	+++	−	−	−
W30	−	−	+	−	−	−	−	−
W31	−	−	+++	−	−	−	−	+
W33	−	−	++	−	−	−	−	−
W36	−	−	+	−	+	−	−	−
W41	−	−	−	−	+++	−	−	−
W42	−	−	−	−	++	−	−	++
W44	−	−	−	−	+++	−	−	−
W47	−	−	+++	−	−	−	−	−
W52	−	−	++	−	+	−	−	+
P1	−	−	+++	−	+++	−	−	+++
P2	−	−	+++	−	++	−	−	+++
P3	−	−	+	−	−	−	−	−
P10	−	−	+++	−	−	−	−	++
P11	−	−	+	−	+++	−	−	++
P14	−	+++	+++	+++	−	−	−	−
P16	+	−	+	−	+++	+	−	+
P18	−	−	+	+	−	−	−	+++
P25	−	−	−	−	+	−	−	+
P27	−	−	−	−	+++	−	−	++
P29	−	−	+++	−	−	−	−	−
P33	−	−	−	+	−	−	−	−
P39	−	+++	+++	−	−	−	−	++
Frequency (%)	3	6	69	13	63	3	0	50

[a] Effector cells were generated by polyclonal in vitro stimulation of PBMC with autologous blast cells, as described in Materials and Methods.

[b] Target cells were autologous EBV-LCL that had been previously pulsed with long peptides, as described in Materials and Methods; target peptides are designated by amino acid positions. Specific chromium release obtained with effector and target cells in ratios ranging from 60:1 to 120:1, depending on the donor, is indicated as follows: +++, >30%; ++, 20 to 29%; +, 10 to 19%; or −, <10%. This 10% cutoff was determined by the observation that nine seronegative donors showed no cytotoxic activity above 10%, under the same protocol.

84-to-91 octapeptide and is different from the highly overlapping epitope associated with HLA-A11.

CTL epitopes in region 113 to 128. We have previously shown that two donors, W2 and W8, recognized epitopes contained in the 115-to-128 Nef sequence. The use of nested peptides derived from peptide 115 to 128 indicated that W2 CTL probably recognized peptide 115 to 125 in association with the HLA-B17 molecule and that W8 CTL recognized peptide 117 to 128 in association not only with the autologous HLA-B37 molecule but also with the heterologous HLA-B17 molecule (6). We extended these previous results by showing that both B57 and B58 molecules, two splits of HLA-B17, functioned with W2 and W8 CTL, since G3-EBV, 44-EBV, and 27-EBV target cells (HLA-$B57^+$) were recognized, as well as M3-EBV and W2-EBV target cells (HLA-$B58^+$) (Fig. 1G and H). Furthermore, a new HLA-restricting molecule, HLA-B62, was defined in the 117-to-128 region, with donors W22 (Fig. 1I), W41, and P1. In the absence of enough PBMC, it was not possible to define these different epitopes more precisely, but the main point is that the 115-to-128 region (14 amino acids) can be recognized in association with three HLA molecules.

CTL epitopes in region 126 to 144. The 126-to-144 region was recognized by seven donors in association with three HLA molecules: P11 with HLA-B49, W8 and W42 with HLA-B7, and P16, W13, W24, and W44 with HLA-B18, as illustrated in Fig. 1J to L for each HLA restriction. The 126-to-138 peptide was recognized with W8 CTL, indicating that the epitope associated with HLA-B7 is included in this sequence (Table 5). In the absence of nested peptides corresponding to this sequence, it was not possible to define this epitope more accurately. On the other hand, the shortest peptide recognized by P11 and W24 CTL was 135 to 143. Titration curve assays evidenced that the 135-to-143 peptide could be recognized by P11 CTL to a final concentration of 10^{-7} μg/ml (i.e., about 10^{-13} M), whereas peptides 134 to 143 and 135 to 144 were recognized only up to concentrations of 10^{-5} and 10^{-3} μg/ml, respectively, strongly suggesting that the 135-to-143 peptide corresponds to the epitope presented by HLA-B49 molecules (Fig. 3). This peptide was also the smaller one recognized in association with HLA-B18, but unfortunately, a lack of corresponding PBMC made us unable to perform a titration curve assay.

DISCUSSION

Extensive study of Nef epitopes recognized by CTL provides us with evidence that epitopes conserved within the HIV-1/

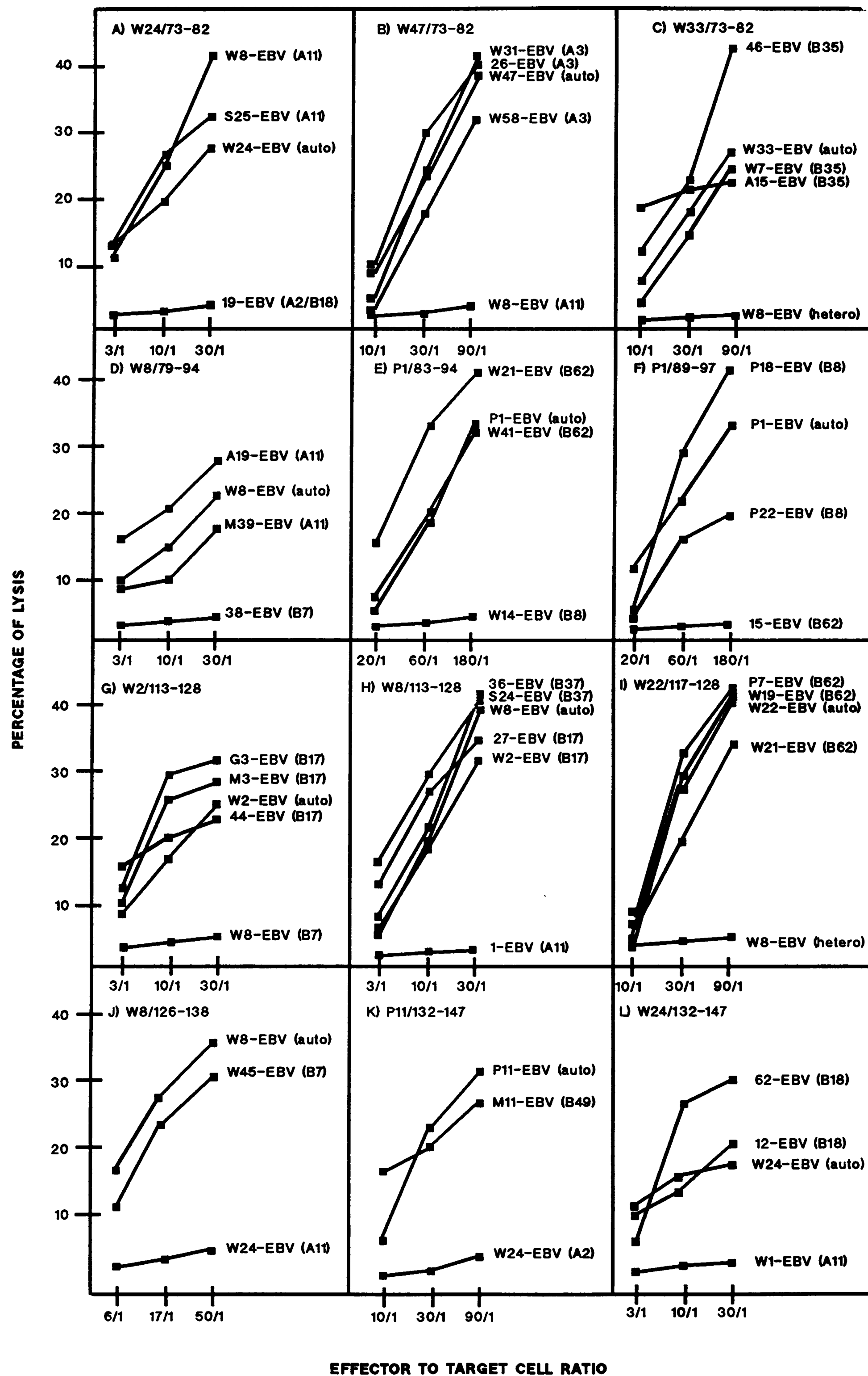

FIG. 1. Recognition of Nef peptides by HIV-1-specific CTL. The effector cells were generated from Nef-responding donors, either in polyclonal cell lines (panels B, C, E, F, I, J, and K) or in antipeptide cell lines (panels A, D, G, H, and L). The peptide used to stimulate antipeptide cell lines was, in each case, the same as the peptide used to sensitize target cells. The CTL-mediated specific lysis of peptide-pulsed targets was calculated after substraction of lysis obtained with the corresponding unpulsed targets. This background lysis was in all cases inferior to 12%. The HLA phenotype of the different targets is indicated in Table 2.

TABLE 2. HLA phenotype of the different target cells

Donor	HLA-A	HLA-B	HLA-C
A15	3/3[a]	27/35	4/4
A19	1/11	8/27	2/—
G3	24/28	17(57)/51	1/4
M3	2/29	17(58)/40(60)	3/—
M11	2/3	35/49	4/—
M39	2/11	27/35	2/4
P1	1/9(24)[b]	8/15(62)	3/7
P7	28/19.2(30)	14/15(62)	3/7
P11	2/32	12(44)/21(49)	2/—
P18	11/29	8/18	4/—
P22	2/2	8/35	4/—
W1	2/9(23)	27/35	1/4
W2	29/9(24)	7/17(58)	6/7
W7	1/2	8/35	2/7
W8	11/26	7/37	6/7
W14	1/28	8/17	4/7
W19	2/19.2	18/15(62)	5/—
W21	2/29	12(44)/15(62)	5/—
W22	28/9(23)	7/15(62)	3/7
W24	2/11	18/—	7/—
W31	1/3	8/35	4/7
W33	10/29	35/12(44)	4/—
W41	2/—[c]	15(62)/27	2/3
W45	3/19(29)	7/8	1/5
W47	3/34	12(44)/70	4/—
W58	3/34	12(44)/47	7/—
S24	1/32	37/40	1/3
S25	2/11	5/37	1/—
1[d]	11	35	4
12[d]	19(30)	18	5
15[d]	2	15(62)	9
19[d]	2	18	5
26[d]	3	7	7
27[d]	2	17(57)	6
36[d]	1	37	6
38[d]	3	7	7
44[d]	2	17(57)	7
46[d]	31(19)	35	4
62[d]	3	18	5

[a] Serological specificities of the two alleles from the corresponding HLA molecule.
[b] Split inside a particular serological specificity.
[c] —, not determined.
[d] Homozygous cell line provided from the Tenth International Histocompatibility Workshop and Conference of 1987.

LAI isolate and isolates from a large number of infected individuals are not equally distributed throughout the Nef protein but are concentrated in three peptide regions (66 to 100, 115 to 146, and 182 to 206). To our knowledge, this is the first observation of such a phenomenon for epitopes recognized in association with HLA class I molecules. Nevertheless, it is possible that CTL epitopes are also present in the other more variable parts of the Nef protein, since the central region is the most highly conserved among HIV-1 isolates. However, we cannot exclude that, by use of Nef peptides corresponding to autologous viral isolates, CTL epitopes could be localized to other regions of the Nef protein.

The present report shows that, while this response is a composite of multiple reactivities against different epitopes within the whole Nef protein, the central region of the molecule, which contains two of the above-described regions, is immunodominant for most of the donors tested. The results presented here also confirm that the recognition of peptide fragments of the Nef protein by HLA is controlled by the immunodominance of some HLA molecules, so that a specific

TABLE 3. $CD8^+$ epitopes present in the 73-to-82 region of Nef

Peptide (amino acid positions)	Sequence	Specific Cr release (%)[a] with indicated donor[b] at indicated effector/target cell ratio					
		W24		W47		W33	
		150/1	50/1	67/1	22/1	70/1	6/1
73–82	QVPLRPMTYK	33	18	38	27	41	12
73–81	QVPLRPMTY	<2	<2	<2	<2	32	17
73–80	QVPLRPMT	<2	<2	<2	<2	7	2
74–82	VPLRPMTYK	7	2	4	3	45	29
74–81	VPLRPMTY	<2	<2	<2	<2	36	25
75–82	PLRPMTYK	5	<2	<2	<2	<2	<2
None		4	<2	4	2	2	<2

[a] Specific chromium release of autologous target cells was calculated as described in Materials and Methods.
[b] Effector cells were generated in polyclonal anti-HIV cell lines as described in Materials and Methods. For donors W24, W47, and, W33, respectively, the possible epitopes were amino acid sequences 73 to 82, 73 to 82, and 74 to 81, and the MHC restrictions were HLA-A11, HLA-A3, and HLA-B35. The presenting HLA molecule was determined by use of different heterologous target cells, as illustrated in Fig. 1.

HLA haplotype might be selected as the major T-epitope-presenting molecule. For example, patient P10 expressed both HLA-B17 and B8 molecules and produced HLA-B8-restricted CTL against peptide 90 to 97 but did not generate a CTL response with a Nef peptide presented by the HLA-B17 haplotype (peptide 117 to 125). Some patients had HLA molecules that recognized several fragments with the same restriction. Thus, the CTL from patient W24 recognized both peptides 73 to 82 and 84 to 92 with an HLA-A11 restriction. A similar multiple HLA-A11-restricted CTL response against nuclear antigen 4 of EBV (EBNA-4) has recently been described (11).

More surprisingly, a specific region was recognized in asso-

TABLE 4. $CD8^+$ epitopes present in the 83-to-97 region of Nef

Peptide (amino acid positions)	Sequence	Specific Cr release (%)[a] with indicated donor[b] at indicated effector/target cell ratio					
		W8[c]		P1[d]		P10[d]	
		60/1	20/1	90/1	30/1	110/1	37/1
83–94	AAVDLSHFLKEK	44	17	39	22	<2	<2
83–91	AAVDLSHF	<2	<2	27	18	<2	<2
84–94	AVDLSHFLKEK	21	11	19	12	<2	<2
84–93	AVDLSHFLKE	27	10	26	23	NT[e]	NT
84–92	AVDLSHFLK	31	17	NT	NT	NT	NT
84–91	AVDLSHF	NT	NT	37	16	NT	NT
85–94	VDLSHFLKEK	<2	<2	<2	<2	NT	NT
86–94	DLSHFLKEK	5	<2	<2	<2	<2	<2
86–97	DLSHFLKEKGG	NT	NT	52	29	42	24
89–97	HFLKEKGG	4	<2	57	41	38	26
90–97	FLKEKGG	NT	NT	43	28	37	19
90–100	FLKEKGGLEGL	NT	NT	34	17	31	21
None		3	<2	4	<2	5	3

[a] Specific chromium release of autologous target cells.
[b] For donors W8 and P10, respectively, the possible epitopes were amino acid sequences 84 to 92 and 90 to 97, and the MHC restrictions were HLA-A11 and HLA-B8. For donor P_1, there are two possible epitopes, 84 to 91 and 90 to 97, recognized in association with HLA-B62 and HLA-B8, respectively. The presenting HLA molecule was determined by use of different heterologous target cells, as illustrated in Fig. 1.
[c] Effector cells were generated in anti-peptide cell lines.
[d] Effector cells were generated in polyclonal anti-HIV cell lines.
[e] NT, not tested.

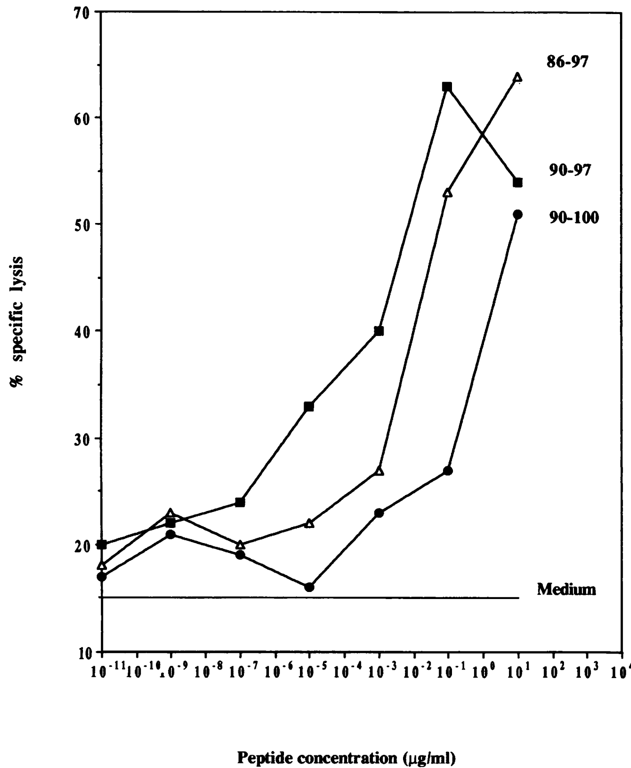

FIG. 2. Dose titration of the 86-to-97, 90-to-97, and 90-to-100 peptides. Autologous target cells were incubated with the indicated concentrations of peptide, and effector cells were generated in polyclonal anti-HIV cell lines from donor P10. The effector-to-target-cell ratio was 80/1.

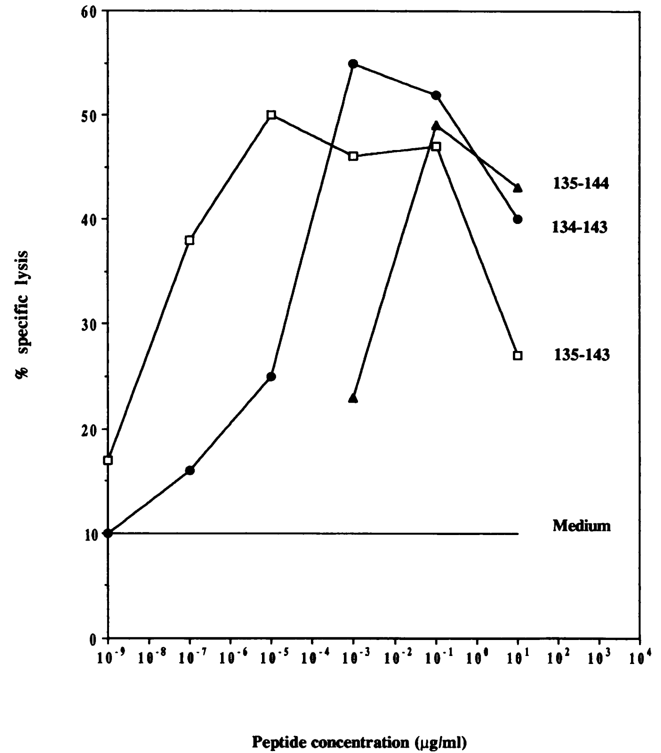

FIG. 3. Dose titration of the 134-to-143, 135-to-144, and 135-to-143 peptides. Autologous target cells were incubated with the indicated concentrations of peptide, and effector cells were generated in polyclonal anti-HIV cell lines from donor P11. The effector-to-target-cell ratio was 90/1.

ciation with several HLA-A or HLA-B molecules. Four antigenic regions encompassing the Nef 73-to-82, 83-to-97, 113-to-128, and 126-to-144 sequences were recognized by different HLA class I molecules, suggesting the existence of multirestriction. A set of 8- to 10-residue peptides were used to determine

TABLE 5. CD8$^+$ epitopes present in the 126-to-144 region of Nef

Peptide (amino acid positions)	Sequence	Specific Cr release (%)[a] with indicated donor[b] at indicated effector/target cell ratio					
		W8		P11		W24	
		130/1	44/1	60/1	20/1	90/1	30/1
126–138	NYTPGPGVRYPLT	46	18	<2	<2	4	<2
132–147	GVRYPLTFGWCYKLVP	<2	<2	48	35	43	28
132–144	GVRYPLTFGWCYK	NT[c]	NT	42	29	NT	NT
134–144	RYPLTFGWCYK	NT	NT	40	26	30	27
134–143	RYPLTFGWCY	NT	NT	45	44	30	15
135–144	YPLTFGWCYK	NT	NT	54	42	37	24
135–143	YPLTFGWCY	NT	NT	40	37	33	27
137–145	LTFGWCYK	NT	NT	7	5	3	<2
None		4	2	2	<2	2	2

[a] Specific chromium release of autologous target cells.
[b] Effector cells were generated in polyclonal anti-HIV cell lines. For donors W8, P11, and W24, respectively, the possible epitopes were amino acid sequences 126 to 138, 135 to 143, and 135 to 143, and the MHC restrictions were HLA-B7, HLA-B49, and HLA-B18 (the presenting HLA molecule was determined by use of different heterologous target cells, as illustrated in Fig. 1).
[c] NT, not tested.

fine CTL specificity. The majority of peptides were in fact recognized only by a single HLA molecule. This fine specificity will be discussed in relation with known CTL epitopes recognized with the same MHC molecule in other viral systems.

We described two HLA-A11-restricted epitopes, Nef 73 to 82 and Nef 84 to 92. A comparison of their sequences with sequence 416 to 424 of EBNA-4 (11), a high-affinity ligand for HLA-A11, indicates that the essential anchoring residues for HLA-A11 seem to be Val in position 2 (Nef 74 to 81, in which Val is in position 1, is inactive) and the C-terminal lysine (in position 9 for the EBNA-4 and Nef 84-to-92 epitopes and in position 10 for the Nef 73-to-82 epitope) (Table 6).

The decameric peptide Nef 73 to 82 was both HLA-A3 and HLA-A11 restricted (Table 2). In view of the complete identity of binding pockets A, B, C, and F and the near identity of pocket D of HLA-A3 and -A11 (9, 22), it is not surprising that the same peptide associates with both molecules. However, peptide Nef 84 to 92 was recognized only with HLA-A11, suggesting that the requirements for functional recognition with a given HLA molecule are not the same for different peptides. The same is true with HLA-B37, which can present like HLA-B17 peptide Nef 120 to 128 but not like peptide 116 to 125.

The octameric peptide Nef 74 to 81 and peptide pp65 123 to 131 of human cytomegalovirus (10) are the two functional epitopes to be described in association with HLA-B35 (Table 6). Interestingly, they shared with peptides eluted from HLA-B35 molecules proline in position 2 and a C-terminal tyrosine (8, 25).

In the same way, the octapeptide Nef 90 to 97, which is

TABLE 6. Comparison of minimal peptides recognized by the same HLA class I molecule

HLA class	Virus	Peptide	Sequence[a]	Source or reference
A3	HIV-1	Nef 73–82	QVPLRPMTYK	This study
	HIV-1	Env 768–778	RLRDLLLIVTR	24
A11	HIV-1	Nef 73–82	QVPLRPMTYK	This study
	HIV-1	Nef 84–92	AVDLSHFLK	This study
	EBV	EBNA-4 416–424	IVTDFSVIK	11
B8	HIV-1	Nef 90–97	FLKEKGGL	This study
	Influenza virus	NP 380–388	ELRSRYWAI	23
	EBV	EBNA-3 339–347	FLRGRAYGI	4
B35	HIV-1	Nef 74–81	VPLRPMTY	This study
	HCMV[b]	pp65 123–131	IPSINVHHY	10

[a] Underlined symbols designate essential anchoring residues.
[b] HCMV, human cytomegalovirus.

recognized in association with HLA-B8, includes the proposed motif with a lysine or an arginine in positions 3 and 5 and a leucine or an isoleucine in the C-terminal position (4, 7, 23) (Table 6).

It seems likely that the decameric sequence 128 to 137 or the octameric sequence 130 to 137 corresponds to the epitope in peptide Nef 126 to 138, which was tested in this study and which was recognized with HLA-B7, since peptides eluted from this HLA molecule often have a proline in position 2 and a C-terminal leucine (14).

Lastly, we were able to evidence for the first time functional peptides which can be recognized in association with HLA-B18, HLA-B49, and HLA-B62 molecules, for the Nef 135-to-142 peptide presented by HLA-B49 dilution experiments allowed us to show that it works at very low concentration, indicating that it probably corresponds to the cognate epitope. Unfortunately, the same assays cannot be done, to date, for the other peptides, which are Nef 153 to 142 with HLA-B18 and Nef 84 to 91 with HLA-B62.

Our use of 8- to 10-residue peptides in a functional CTL assay enabled us to show that a peptidic sequence of about 70 amino acids can be recognized by numerous MHC class I molecules. In view of the wide range of HLA molecules used as restricting elements by Nef-specific CTL (13 different HLA molecules; see references 13 and 15 and this study), and taking into account the frequency of the different HLA haplotypes, this particular region should be able to elicit a CTL response in almost the entire Caucasian population. Work from our laboratory has recently demonstrated the possibility of inducing specific CTL in vivo by immunization of mice (17) or macaques (2) with long lipopeptides, including MHC class I-restricted epitopes from HIV-1 Env protein or simian immunodeficiency virus Nef and Gag proteins. These results suggest that a vaccination protocol may be designed with the most conserved epitopes in order to induce an efficient $CD8^+$ T-cell response. The Nef protein is particularly interesting for this, since CTL directed against this regulatory, early-expressed protein could lead to the elimination of infected cells before the release of new viral particles. However, it is probably essential to combine CTL induction with a strong, persistent neutralizing antibody activity.

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REFERENCES

1. **Bodmer, H. C., F. M. Gotch, and A. J. McMichael.** 1989. Class I-restricted T cells reveal a low responder allele due to processing of viral antigen. Nature (London) **337:**653–656.
2. **Bourgault, I., F. Chirat, A. Tartar, J.-P. Lévy, J.-G. Guillet, and A. Venet.** 1994. Simian immunodeficiency virus as a model for vaccination against HIV. Induction in rhesus macaques of GAG- or NEF-specific cytotoxic T lymphocytes by lipopeptides. J. Immunol. **152:**2530–2537.
3. **Bourgault, I., A. Gomez, E. Gomard, and J.-P. Lévy.** 1991. Limiting-dilution analysis of the HLA restriction of anti-Epstein-Barr virus-specific cytolytic T lymphocytes. Clin. Exp. Immunol. **84:**501–507.
4. **Burrows, S. R., S. J. Rodda, A. Suhrbier, H. M. Geysen, and D. J. Moss.** 1992. The specificity of recognition of a cytotoxic T lymphocyte epitope. Eur. J. Immunol. **22:**191–195.
5. **Culmann, B., E. Gomard, M.-P. Kiény, B. Guy, F. Dreyfus, A.-G. Saimot, D. Sereni, and J.-P. Lévy.** 1991. Six epitopes reacting with human cytotoxic $CD8^+$ T cells in the central region of the HIV-1 NEF protein. J. Immunol. **146:**1560–1565.
6. **Culmann, B., E. Gomard, M.-P. Kiény, B. Guy, F. Dreyfus, A.-G. Saimot, D. Sereni, D. Sicard, and J.-P. Lévy.** 1989. An antigenic peptide of the HIV-1 NEF protein recognized by cytotoxic T lymphocytes of seropositive individuals in association with different HLA-B molecules. Eur. J. Immunol. **19:**2383–2386.
7. **DiBrino, M., K. C. Parker, J. Shiloach, R. V. Turner, T. Tsuchida, M. Gargield, W. E. Biddison, and J. E. Coligan.** 1994. Endogenous peptides with distinct amino acid anchor residue bind to HLA-A11 and HLA-B8. J. Immunol. **152:**620–631.
8. **Falk, K., O. Rötzschke, B. Grahovac, D. Schendel, S. Stevanovic, G. Jung, and H.-G. Rammensee.** 1993. Peptide motifs of HLA-B35 and -B37 molecules. Immunogenetics **38:**161–162.
9. **Garrett, T. P. J., M. A. Saper, P. J. Björkman, J. L. Strominger, and D. C. Wiley.** 1989. Specificity pockets for the side chains of peptide antigens in HLA-Aw68. Nature (London) **342:**692–695.
10. **Gavin, M. A., M. J. Gilbert, S. R. Ridell, P. D. Greenberg, and M. J. Bevan.** 1994. Alkali hydrolysis of recombinant proteins allows for the rapid identification of class I MHC-restricted CTL epitopes. J. Immunol. **151:**3971–3980.
11. **Gavioli, R., M. G. Kurilla, P. O. de Campos-Lima, L. E. Wallace, R. Dolcetti, R. J. Murray, A. B. Rickinson, and M. G. Masucci.** 1993. Multiple HLA A11-restricted cytotoxic T-lymphocyte epitopes of different immunogenicities in the Epstein-Barr virus-encoded nuclear antigen 4. J. Virol. **67:**1572–1578.
12. **Gomez, A., I. Bourgault, E. Gomard, F. Picard, and J.-P. Lévy.** 1989. Role of different subsets in human anti-viral cell cultures. Cell. Immunol. **118:**312–327.
13. **Hadida, F., A. Parrot, M.-P. Kiény, B. Sadat-Sowti, C. Mayaud, P. Debré, and B. Autran.** 1992. 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. J. Clin. Invest. **89:**53–60.
14. **Huczko, E. L., W. M. Bodnar, D. Benjamin, K. Sakaguchi, N. Zhou Zhu, J. Shabanowitz, R. A. Henderson, E. Appella, D. F. Hunt, and V. H. Engelhard.** 1993. Characteristics of endogenous peptides eluted from the class I molecule HLA-B7 determined by mass spectrometry and computer modeling. J. Immunol. **151:**2572–2587.
15. **Jassoy, C., T. Harrer, T. Rosenthal, B. A. Navia, J. Worth, R. P. Johnson, and B. D. Walker.** 1993. Human immunodeficiency type 1-specific cytotoxic T lymphocytes release gamma interferon, tumor necrosis factor alpha (TNF-α), and TNF-β when they encounter their target antigens. J. Virol. **67:**2844–2852.
16. **Lamhamedi-Cherradi, S., B. Culmann-Penciolelli, B. Guy, M.-P.**

Kiény, F. Dreyfus, A.-G. Saimot, D. Sereni, D. Sicard, J.-P. Lévy, and E. Gomard. 1992. Qualitative and quantitative analysis of human cytotoxic T-lymphocyte responses to HIV-1 proteins. AIDS **6**:1249–1258.

17. **Martinon, F., H. Gras-Masse, C. Boutillon, F. Chirat, B. Deprez, J.-G. Guillet, E. Gomard, A. Tartar, and J.-P. Levy.** 1992. Immunization of mice with lipopeptides bypasses the prerequisite for adjuvant. Immune response of BALB/C mice to human immunodeficiency virus envelope glycoprotein. J. Immunol. **149**:3416–3422.
18. **Myers, G., J. A. Berzofsky, B. Korba, R. F. Smith, and G. N. Pavlakis (ed.).** 1992. Human retrovirus and AIDS. Los Alamos Laboratory, Los Alamos, N.Mex.
19. **Nixon, D. F., A. R. M. Townsend, J. G. Elvin, C. R. Rizza, J. Gallway, and A. J. McMichael.** 1988. HIV-1 gag-specific cytotoxic T lymphocytes defined with recombinant vaccinia virus and synthetic peptides. Nature (London) **336**:484–487.
20. **Oldstone, M. B. A., A. Tishon, R. Geckeler, and H. Lewicki.** 1992. A common antiviral cytotoxic T-lymphocyte epitope for diverse histocompatibility complex haplotypes: implication for vaccination. Proc. Natl. Acad. Sci. USA **89**:2752–2758.
21. **Rötzschke, O., and K. Falk.** 1991. Naturally-occurring peptide antigens derived from the MHC class-I-restricted processing pathway. Immunol. Today **12**:447–455.
22. **Saper, M. A., P. Björkman, and D. C. Wiley.** 1991. Refined structure of the human histocompatibility antigen HLA-A2 at 2.6 A resolution. J. Mol. Biol. **219**:277–319.
23. **Sutton, J., S. Rowland-Jones, W. Rosengerg, et al.** 1993. A sequence pattern for peptides presented to cytotoxic T lymphocytes by HLA-B8 revealed by analysis of epitopes and eluted peptides. Eur. J. Immunol. **23**:447–453.
24. **Takahashi, K., L.-C. Dai, T. R. Fuerst, W. E. Biddison, P. L. Earl, B. Moss, and F. A. Ennis.** 1991. Specific lysis of human immunodeficiency virus type 1-infected cells by a HLA-A3.1-restricted CD8 cytotoxic T-lymphocyte clone that recognizes a conserved peptide sequence within the gp41 subunit of the envelope protein. Proc. Natl. Acad. Sci. USA **88**:10277–10281.
25. **Takamiya, Y., C. Schönbach, K. Nokihara, M. Yamaguchi, S. Ferrone, K. Kano, K. Egawa, and M. Takaguchi.** 1993. HLA-B*3501-peptide interactions: role of anchor residues of peptides in their binding to HLA-B*3501 molecules. Int. Immunol. **6**:255–261.
26. **Townsend, A. R. M., J. Rothbard, F. M. Gotch, G. Baharur, D. Wraith, and A. J. McMichael.** 1986. The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. Cell **44**:959–968.