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Recognition of a Small Number of Diverse Epitopes Dominates the Cytotoxic T Lymphocyte Response to HIV Type 1 in an Infected Individual

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ABSTRACT

Mitogen-activated T cell lines may be reproducibly used to identify relatively conserved HIV-1 epitopes that dominate CTL recognition of HIV-infected cells. Using a combination of nested truncations of HIV–vaccinia recombinants encoding HIV-1_{LAI} Env and overlapping peptides that span the coding regions of the HIV-1 SF2 subclone of *env*, *gag*, *nef*, *rev*, and *tat*, we have mapped the immunodominant, relatively conserved CTL epitopes recognized by 25 HIV-seropositive individuals with CD4 counts between 100 and 500/mm^3 and no history of AIDS opportunistic infection. We could characterize at least 1 peptide CTL epitope recognized by the T cell lines of 18 of 25 of the subjects; the T cell lines from 2 additional subjects recognized HIV–vaccinia presenting targets, but no dominant peptide epitope was identified. CTL epitopes were most frequently encoded by *gag* (recognized by 16 of 25 patient T cell lines), followed by *nef* and *env* (11 of 25 each), and the RT region of *pol* (9 of 25). Tat and Rev were rarely the sites of CTL epitopes. The identified epitopes occurred predominantly in relatively conserved regions of HIV-1. The mean number of HIV peptides identified at a single time for each cell line was 2.7 ± 1.7. Although no single peptide dominated CTL recognition in more than four individuals, clusters of epitopes were found in the N-terminal region of gp160 and in two central regions of Nef. The dominant HIV-1 CTL epitopes in infected patients were not predictable on the basis of MHC expression and varied widely in an MHC-diverse population.

INTRODUCTION

HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 infection stimulates an unusually strong cytotoxic T lymphocyte (CTL) response that declines late in disease.[1–3] CTLs are important in clearing or controlling many intracellular infections and appear to be prominent in the resolution of the viremia of acute HIV-1 infection.[4,5] Because of the exceptionally high precursor frequency of HIV-specific CTLs circulating in the blood of HIV-1-infected individuals, it is possible using peripheral blood mononuclear cell (PBMC)-derived T cell lines to identify HIV-encoded epitopes, whose recognition dominates the CTL response to the virus. In previous work we found that CTL recognition of HIV-1_{LAI} gp160 in infected individuals is dominated by the recognition of a few peptide epitopes.[6] We now report the results of HIV-1 gp160, Gag, Nef, Tat, and Rev CTL epitope determination in 25 HIV-seropositive persons with CD4 counts of 100–500/mm^3 and no history of opportunistic infections or AIDS-related neuropathy. One purpose of this study is to determine whether a small or large number of HIV epitopes is recognized. The fewer epitopes are recognized, the more likely the virus might be able to mutate to escape CTL recognition. Another goal of this study is to identify regions of HIV that might encode epitopes recognized by many individuals for possible incorporation into vaccine or immunotherapeutic strategies.

Because we use target cells that express the HIV genes of laboratory strains or are incubated with laboratory strain-derived peptides, we are unable to detect CTL epitopes that may be specific to the quasispecies of HIV infecting a particular patient. However, epitopes in variable regions of HIV may be less important for protection or for immunotherapy since the virus should be able to mutate in these regions to escape CTL recognition. Our method may also miss epitopes that are cryptic or weakly recognized compared to the immunodominant epitopes. These could become significant

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at another time in the disease course or might become important with specific therapeutic intervention.

HIV infection can serve as a prototype to study the diversity of CD8 T cell antigen recognition at both the individual and population level. Similar studies of CTL recognition in MHC-diverse populations have not been done for other pathogens.

MATERIALS AND METHODS

Subjects

Subjects were HIV-1-seropositive patients, enrolled in the Division of AIDS Treatment Research Initiative (DATRI) Protocol 006, with CD4 counts between 100 and 500/mm^3 without a history of AIDS-defining opportunistic infections in the General Medical or Infectious Diseases Clinics of New England Medical Center (Boston, MA) (Table 1). Informed consent was obtained from each subject. This study was approved by the New England Medical Center Human Investigation Review Committee.

Cell lines

T cell lines were generated by adding phytohemagglutinin (PHA-P, 2 μg/ml; Difco, Detroit, MI) to PBMCs obtained by Ficoll-Hypaque density centrifugation from heparinized blood. Cells were incubated at 5×10^5/ml in T cell medium (RPMI 1640 supplemented with 15% fetal calf serum [JRH Biosciences, Lenexa, KS], recombinant human interleukin 2 [rhuIL-2, 200 U/ml; Cetus, Emeryville, CA], 2 mM glutamine, 2 mM HEPES, penicillin [100 U/ml], streptomycin [100 μg/ml], and 50 μM 2-mercaptoethanol). Twice a week the cultures were adjusted to 5×10^5/ml with fresh T cell medium. PBMCs for some of the experiments were thawed from samples previously frozen to liquid N_2 in 5% dimethyl sulfoxide (DMSO), 95% fetal calf serum using a programmed cell freezer (Gordinier, Roseville, MI). Autologous B lymphoblastoid cell lines (B-LCLs) were generated for each subject, using standard methods.

Vaccinia vectors

Vaccinia vectors encoding LacZ (vSC8), Env of the BH8 isolate of HIV-1_{IIIB} (vPE16), Gag of the HXB.2 subclone (vDK1), Tat (vtat), and all but the last 22 residues of HXB.2 RT (vCF21) were used to screen cell lines for specific cytotoxicity against gp160, Gag, Tat, and RT-expressing targets.[1,7–9] A set of vaccinia vectors expressing nested truncations of the same Env sequence was used to localize potential regions containing dominant Env epitopes (vPE17, vPE18, vPE8, vPE20, vPE21, vPE22 as previously described).[6,10]

Peptides

Overlapping 22-mers spanning amino acids 748–863 (amino acid numbering as in Ref. 11) of the BH8 isolate of HIV-1_{IIIB} with

TABLE 1. PERCENT SPECIFIC CYTOTOXICITY OF T CELL LINES FROM 25 HIV-SEROPOSITIVE INDIVIDUALS AGAINST AUTOLOGOUS B-LCL TARGETS INFECTED WITH HIV$_{LAI}$–VACCINIA RECOMBINANTS EXPRESSING Env, Gag, Tat, AND THE RT REGION OF Pol OR LacZ CONTROL[a]

Patient	CDC disease stage	CD4 count (cells/mm^3)	HLA class I		Percent specific cytotoxicity[b] (E:T ratio 25:1)				
			A	B	Env	Gag	RT	Tat	LacZ
201	A2	470	*3, 32*	*7, 14*	**28**	**23**	16	9	12
202	A2	400	*Nd*	*Nd*	**18**	12	16	11	8
203	B2	360	*3, 24*	*8, 55*	**20**	**17**	12	5	7
204	A2	440	*2, 3*	*8, 62*	**16**	**18**	**19**	Nd	6
211	A2	240	*1, 24*	*44, 62*	20	12	12	10	12
214	B2	360	*2, –*	*21, –*	**36**	**33**	12	16	14
215	B3	150	*2, –*	*18, 35*	13	12	10	12	9
216	B2	310	*2, 3*	*14, 44*	**21**	**35**	**24**	4	8
219	A2	240	*2, 24*	*13, 35*	23	**44**	**32**	14	15
221	A3	150	*1, 2*	*50, 57*	6	**23**	5	4	1
222	B2	350	*1, –*	*8, 18*	13	**19**	14	4	8
224	A3	180	*1, –*	*7, 8*	10	9	6	5	4
225	B3	100	*1, 11*	*8, 35*	8	13	7	6	9
226	C3	130	*11, 24*	*8, 53*	11	**19**	17	8	9
228	A2	250	*3, 24*	*7, 38*	**27**	**29**	**39**	9	11
229	A2	330	*2, 33*	*17, –*	16	10	8	**26**	9
230	A2	250	*1, 11*	*8, 27*	8	**20**	4	6	3
231	B2	350	*1, 2*	*8, 14*	10	**38**	8	5	6
233	A3	100	*1, 28*	*13, 44*	44	41	**57**	Nd	35
234	A2	320	*3, 32*	*51, 62*	**49**	**60**	**56**	25	35
235	B3	130	*1, 3*	*7, 15*	**23**	**27**	**27**	12	3
236	A2	270	*2, 24*	*18, 35*	18	10	15	5	9
237	B2	260	*2, 26*	*7, 38*	**22**	13	10	8	4
307	B2	320	*1, 2*	*51, 57*	**33**	**25**	**48**	16	14
322	A1	500	*26, 30*	*38, –*	21	**36**	**27**	Nd	13

[a]The data are presented for an $E:T$ ratio of 25:1, although $E:T$ ratios of 12.5 and 50:1 were also tested. Nd, Not determined.
[b]Values for which the percent specific cytotoxicity against HIV-presenting targets are at least 10% more than control cytotoxicity are set in boldface.

8-amino acid overlaps were synthesized on a Milligen 9050 synthesizer (Bedford, MA). Overlapping 20-mers with 10-amino acid overlaps spanning amino acids 37–851 of the HXB.2 subclone of LAI or of SF2 Env and of SF2 $p24^{gag}$ were provided by the MRC AIDS Reagent Project. Overlapping 15 to 17-mers with 5-amino acid overlaps from SF2 $p15^{gag}$ and $p17^{gag}$ were also provided by the MRC. In some cases an N-terminal cysteine was added to aid in the synthesis. Overlapping 20-mers with 10-amino acid overlaps for *rev*, *nef*-, and *tat*-encoded proteins were synthesized by the European Vaccine against AIDS (EVA) Programme and provided by the MRC. All of the MRC peptides were analyzed by fast atom bombardment mass spectroscopy and high-performance liquid chromatography (HPLC) and found to be of expected mass and greater than 80% pure. Peptide stock solutions (1 mg/ml in 10% DMSO, PBS) were diluted in medium for cytotoxicity assays.

Chromium release assay

Cytotoxicity assays were performed against B-LCL target cells that were either untreated, preincubated with relevant peptides, or infected with vaccinia constructs encoding complete or truncated HIV-1 proteins. For infection, vaccinia virus (2–10 plaque-forming units [PFU]/cell) was added to 5×10^5 exponentially growing B cells in 500 μl in a 24-well plate. The plate was incubated at 37°C over CO_2 with rocking for 1 hr. After 16 hr the cells were harvested and labeled with ^{51}Cr. Infected or uninfected target cells were pelleted and resuspended in 200 μl of serum-containing medium to which 200 μCi of $Na_2(^{51}CrO_4)$ (NEN-Du Pont, Boston, MA) was added. After incubation for 1 hr at 37°C with occasional mixing, the targets were washed three times and resuspended at 10^5/ml. Labeled targets (10^4) were added to triplicate wells of U-bottom microtiter plates. For peptide experiments, the radiolabeled targets were incubated with peptide at a final concentration of 50 μg/ml for 30 min at 37°C over CO_2 and unbound peptide was not removed before adding effector cells. Effector cells after 20–40 days in culture were suspended at various effector-to-target (*E:T*) ratios in 100 μl and added to target cells and the plates incubated at 37°C over CO_2 for 4 hr. For each target, spontaneous release (SR) was determined from wells to which 100 μl of medium was added and total release (TR) calculated from wells containing 100 μl of 1% Nonidet P-40 (NP-40). Supernatants were counted on a Packard Topcount (Meriden, CT) microplate reader. Percent specific cytotoxicity was calculated from the average counts per minute (cpm) as [(average cpm − SR)/(TR − SR)] × 100. Spontaneous release was below 20% of total release. Peptide-specific cytotoxicity was defined as the difference between the percent specific cytotoxicity in the presence or absence of peptide.

HLA typing

Peripheral blood lymphocytes were typed for HLA class I antigen by a standard complement-mediated cytotoxicity assay in the Immunology Laboratory of New England Medical Center.

RESULTS

Cytotoxic T lymphocyte recognition of HIV-1 gp160, Gag, reverse transcriptase, and Tat proteins

Mitogen-stimulated T cell lines (>80% $CD8^+$) from 25 subjects without AIDS-defining illness (except for limited cutaneous Kaposi's sarcoma in subject 226) were tested for their ability to lyse autologous B-LCLs infected with vaccinia recombinants expressing $HIV_{IIIB/LAI}$ gp160, Gag, reverse transcriptase (RT), and Tat proteins or a *lacZ* gene product. If the percent specific lysis of the HIV-expressing target was at least 10% greater than the LacZ-expressing control, then the cell line was defined as cytolytic for cells expressing that HIV protein. Ten percent is well above the background of less than 5% and has been reproducibly associated with our ability to define peptide epitopes.[6] This cutoff has also been used by other researchers.[12,13] T cell lines from 20 of 25 subjects (80%) were cytolytic for at least 1 of these 4 HIV proteins—16 (64%) recognized Gag, 11 (44%) recognized gp160, 9 (36%) recognized RT, and 1 (4%) recognized Tat (Table 1). The 5 T cell lines that did not recognize any of the HIV–vaccinia-infected target cells also did not recognize autologous target cells incubated with overlapping 20-mer peptides that spanned the Nef and Rev proteins. The mean CD4 count of individuals who had T cells cytolytic for at least one HIV protein (301 ± 110) was higher than for those who did not (188 ± 68), but the difference was not significant.

Cell lines that lysed Env-expressing targets at least 10% more than control targets were studied for their lysis of a set of Env truncations followed by assay for lysis of targets incubated with the peptide set spanning the region defined by the truncation.[6,14–16] Cell lines that lysed Gag- or Tat-expressing targets were analyzed for lysis by a set of $p15^{gag}$, $p17^{gag}$, and $p24^{gag}$ peptides or eight Tat peptides, respectively. All cell lines were tested for lysis of the 20 Nef and 11 Rev overlapping peptides. Samples of typical epitope mapping experiments for two subjects are given in Figs. 1 and 2. Of the 18 subjects for whom peptides were identified, the mean number of peptides identified was 2.7 ± 1.7 (Table 2). CTLs from only one subject (214) recognized more than four peptides and they recognized nine (if overlapping peptides were counted once).

Recognition of Env and Gag cytotoxic T lymphocyte epitopes

Of the 11 subjects whose CTLs lysed gp160 expressed from vaccinia recombinant-infected targets, we were able to define peptide epitopes for 7 (Table 2). CTLs from these seven subjects recognized nine distinct peptides: six in gp120 and three in gp41. All the Env epitopes were in relatively conserved regions. Only one peptide was recognized by the CTLs of more than one subject: the amino acid (aa) 49–68 peptide was recognized as dominant by CTLs from three subjects. (This peptide was also weakly recognized by subject 203 below the threshold defined for this article, but recognition was unambiguously present when the T cell line was selectively expanded with the aa 49–68 peptide.[17]) The aa 49–68 peptide contains at least three separate epitopes restricted by A24, B38, and B55 recognized by CTLs from these four subjects[17] and an additional A3 epitope in this region has also been described in a vaccinated HIV-seronegative donor.[18] Single individuals also recognized gp41 peptides that are parts of previously reported multiply restricted clusters at the extreme C terminus[6] and in the region of aa 591–600.[17,19,20]

Sixteen subjects (67% of the total or 89% of those with HIV-specific cytolytic activity above the threshold) recognized vac-

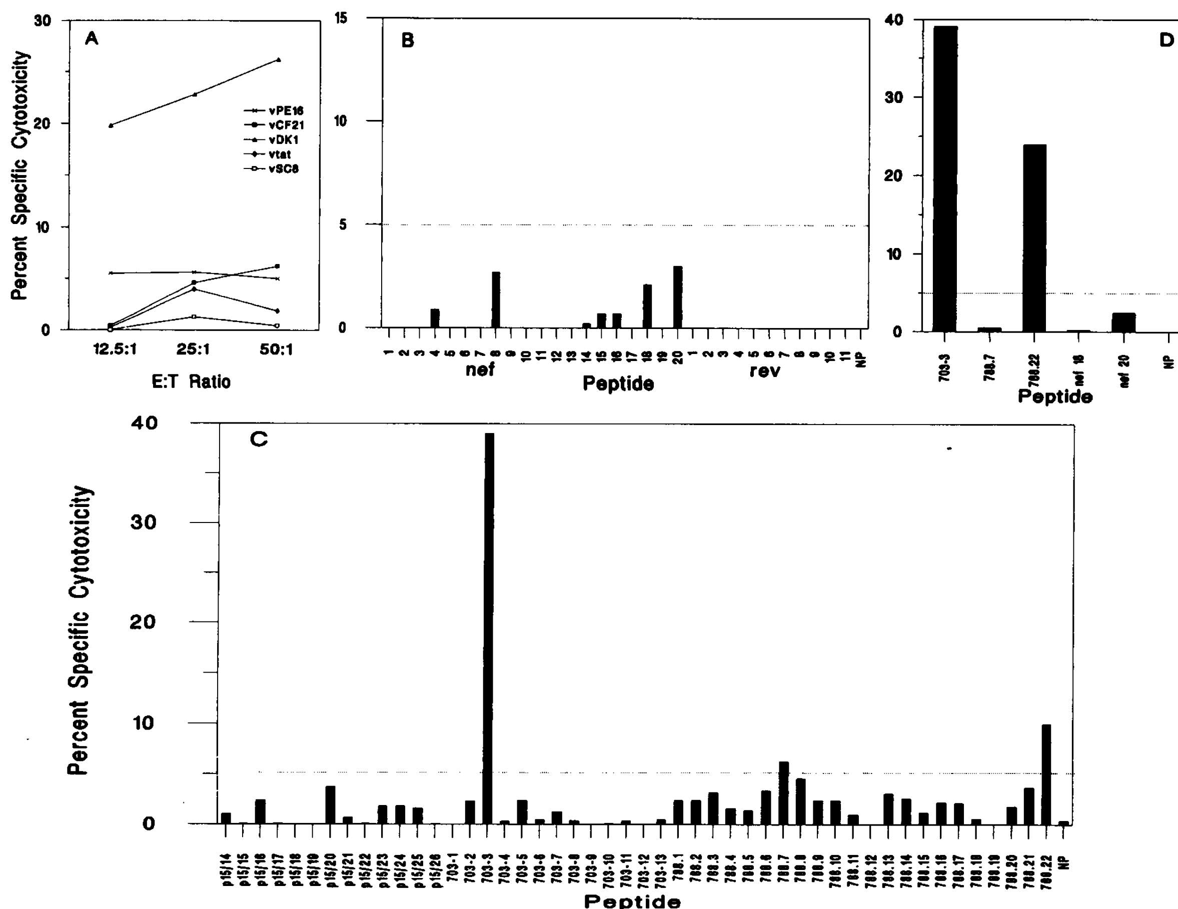

FIG. 1. Epitope mapping for T cell line 221 identified Gag CTL epitopes in p17 (703-3) and p24 (788-22). In an initial screening cytotoxicity assay against HIV–vaccinia recombinant virus-infected autologous cells (A), only Gag-expressing targets (vDK1) were recognized above LacZ-expressing targets (vSC8). Vaccinia virus vPE16 encodes gp160, vCF21 encodes RT, vDK1 encodes Gag, and vtat encodes Tat. Direct assay of peptides from Nef and Rev (B) revealed no specific reactivity. Three Gag peptides (703-3, 788.7, and 788.22) were recognized at least 5% more than no peptide (NP). A subsequent experiment (D) confirmed the 703-3 and 788.22 epitopes. An additional confirmation experiment had similar results and selective expansion of CTLs after exposure to the epitope peptides also confirmed the assignment (data not shown). Peptide data are shown at an *E:T* ratio of 25:1.

cinia–*gag*-expressing autologous targets and peptide epitopes could be identified for 12 of these. Four T cell lines lysed cells expressing a single dominant Gag peptide and eight recognized two Gag peptides. The p24 capsid protein was a target for CTL recognition by all but 1 of the cell lines and contained 11 distinct recognized 20-mers. Although most of the peptides were recognized only once, two peptides (Gag 788.5 [aa 173–192, SALSEGATPQDLNTMLNTVG] and Gag 788.20 [aa 323–342, VQNANPDCKTILKALGPAAT]) were recognized by the CTLs of three subjects and one peptide (Gag 788.13 [aa 253–272, NPPIPVGEIYKRWIILGLNK]) was recognized by CTLs from two subjects (Fig. 3). These three peptides contain previously reported CTL epitopes. Gag 788.5 contains the B14-restricted epitope DLNTMLNTV[15,19] and the three B14-expressing subjects (201, 216, 231) all had CTls that recognize the larger 20-mer. Moreover, a 24-mer containing this region (aa 170–193) has been reported to be recognized by 5 of 14 Gag-specific CTL lines from HIV-seropositive donors.[21] The Gag 788.13 peptide contains a cluster of previously reported MHC-restricted epitopes: B35-restricted PPIPVGDIY,[22] B8-restricted GEIYKRWII,[23] and B27-restricted KRWIILGLNK.[15,24] The two subjects (204 and 222) whose CTLs respond to this peptide express B8 but CTLs from six additional B8-expressing subjects did not respond to Gag 788.13. The Gag 788.20 peptide contains a reported B8-restricted epitope DCKTILKAL[23] and the three T cell lines (203, 230, and 231) that respond to Gag 788.20 all express B8.

The P17 matrix protein was recognized less commonly than p24^{gag}; four CTL lines recognized three distinct 15-mer peptides. The p17 peptide 703/3 (aa 91–105, [C]LRPGGKKKYKLYHIV) recognized by two subjects (219 and 221) contains previously identified epitopes restricted by B8 (GGKKKYKL)[25] and Bw62 (RLRPGGKKKY).[26] These two subjects do not express either of these alleles. None of the T cell lines recognized any of the thirteen 15 to 17-mer overlapping peptides with 5-aa overlaps spanning p15^{gag}.

Recognition of Nef cytotoxic T lymphocyte epitopes

To study the Nef-specific CTL response, we analyzed CTL recognition of a set of 20 overlapping Nef 20-mers directly for

each subject. Eleven subjects (44%) recognized at least 1 Nef peptide. The recognition of Nef was heavily clustered (Table 2, Fig. 3). Of 17 distinct epitopes (where overlapping epitopes are counted as 1), there were 2 clusters: 7 epitopes lay between aa 61 and 100 and 7 epitopes lay between aa 101 and 140. By a χ^2 test this clustering was highly significant ($p < 0.001$). Clusters of multiply restricted Nef CTL epitopes in the central regions of Nef, similar to our finding, were identified at aa

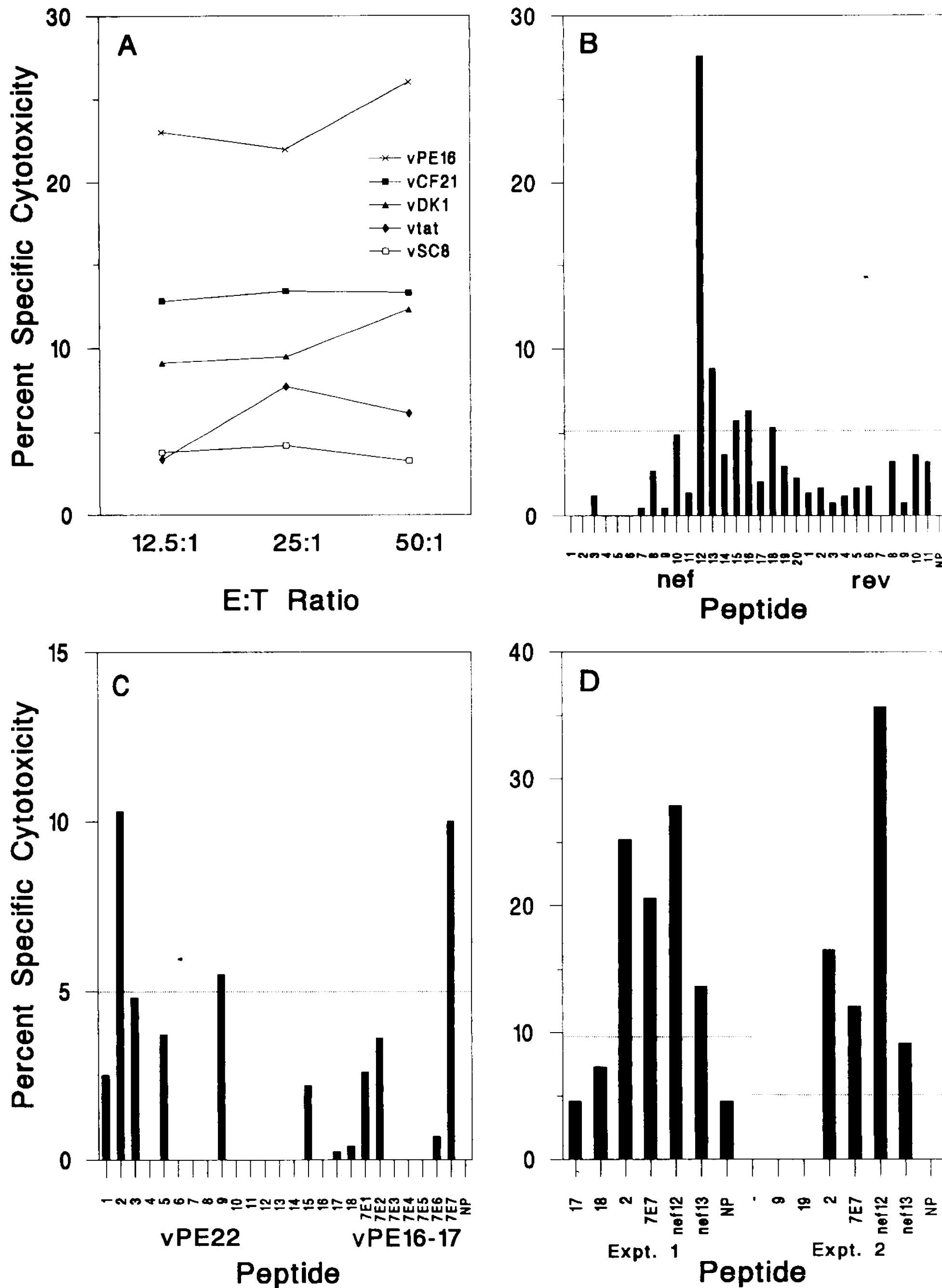

FIG. 2. Epitope mapping for T cell line 237 identified peptide epitopes in gp160 and Nef. In an initial screening assay (A), there was significant cytotoxicity against gp160-expressing targets (vPE16), but not RT (vCF21), Gag (vDK1), or Tat (vtat) above control (vSC8). Direct assay of Nef and Rev peptides revealed potential epitopes [>5% above no peptide (NP) control indicated by dotted line in (B)–(D)] at Nef 12, 13, 15, 16, and 18 but only the epitopes in Nef 12 and 13 were confirmed in subsequent experiments [two of which are shown in (D)]. Cytotoxicity assay with vaccinia–gp160 recombinants (data not shown) localized the epitope to the region between vPE16 and vPE17 and to the region of vPE22. Envelope peptides 740-2 and 740-9 in vPE22 and 7E7 at the C terminus were potentially recognized (C) but only 740-2 and 7E7 were confirmed (D). The *E:T* ratio for the peptide experiments is 25:1. The NP background was higher for the first experiment shown in (D) because the T cells had been in culture for less time.

TABLE 2. IMMUNODOMINANT HIV CTL EPITOPES RECOGNIZED BY 18 OF 20 PATIENTS[a]

Patient no.	*Env peptide*	*Gag peptide*	*Regulatory peptide*
201	env701-60(gp41): aa591-599 ERYLKDQQL	gag788.5(p24): aa173-192 SALSEGATPQDLNTMLNTVG	
202	env740-18(gp120):aa209-228 TQACPKVSFEPIPIHYCAPA		nef 778-7: aa61-80 EEEEVGFPVTPQVPLRPMTY nef 778-17:aa161-180 TSLLHPVSLHGMDDPEREVL
203	No clear dominant peptide despite env recognition	gag703/10 (p17): aa91-105 RIDVKDTKEALEKIE gag788.20 (p24): aa323-342 VQNANPDCKTILKALGPAAT	
204	env740-2(gp120):aa49-68 VPVWKEATTTLFCASDAKAY	gag788.13(p24): aa253-272 NPPIPVGEIYKRWIILGLNK gag788.17(p24): aa293-312 FRDYVDRFYKTLRAEQASQD	nef778-11: aa101-120 HSQRRQDILDLWIYHTQGYF
214	env740-3(gp120):aa59-78 LFCASDAKAYDTEVHNVWAT env740-4(gp120):aa69-88 DTEVHNVWATHACVPTDPN env740-9(gp120):aa119-139 WDQSLKPCVKLTPLCVSLK env740-17(gp120):aa199-219 SLTSCNTSVITQACPKVSFE env740-22(gp120):aa249-268 VSTVQCTHGIRPVVSTQLLL	gag788.3(p24): aa153-172 NAWVKVVEEKAFSPEVIPMF gag788.4(p24): aa163-182 AFSPEVIPMFSALSEGATPQ gag788.8(p24): aa 203-222 ETINEEAAEWDRVHPVVHAGP	nef778-11:aa101-120 HSQRRQDILDLWIYHTQGYF nef778-12:aa111-130 LWIYHTQGYFPDWQNYTPGPGV nef778-13:aa121-140 PDWQNYTPGPGVRYPLTFGW nef778-18:aa171-190 GMDDPEREVLEWRFDSRLAF
216	No clear dominant peptide despite env recognition	gag788.5(p24): aa173-192 SALSEGATPQDLNTMLNTVG	
219		gag703/3(p17): aa91-105 CLRPGGKKKYKLKHIV gag788.10(p24): aa223-242 IAPGQMREPRGSDIAGTTST	rev780-2:aa11-30 LLKAVRLIKFLYQSNPPPNF
221		gag703/3(p17): aa91-105 LRPGGKKKYKLKHIV gag788.22(p24): aa343-362 LEEMMTACQGVGGPGHKARVL	
222		gag788.13(p24): aa253-272 NPPIPVGEIYKRWIILGLNK	

[a]No peptide epitope could be identified for subject 229, whose CTLs recognized vaccinia–*tat*-infected targets or for subject 233, whose CTLs recognized only RT, for which no peptides were available. Epitope mapping was performed using overlapping 15- to 20-mer peptides spanning SF2 Env, Gag, Nef, Rev, and Tat. Patients whose T cells recognize 2 adjacent overlapping peptides probably recognize a single peptide in the 10-aa overlap region. Amino acid numbering as in Ref. 11. The peptides are numbered according to the MRC and EVA Reagent Programmes.

73–94 and 113–147.[27] Of 14 distinct Nef epitope-MHC pairs that have been reported, 11 fall within these 2 central regions and the remaining 3 are within 25 aa of the C terminus, where we have also identified epitopes recognized by single T cell lines.[27–29] These results together with ours suggest that there are two central regions of Nef and a region of lesser importance at the C terminus that dominate CTL recognition of the Nef protein.

Cytotoxic T lymphocyte recognition of Tat and Rev is uncommon

Only one subject (229) has measurable CTL activity against Tat-expressing targets but we were unable to identify a dominant epitope with a set of eight Tat peptide 20-mers with 10-aa overlaps. Rev CTL activity was analyzed directly with a similar set of 11 overlapping Rev 20-mers. T cells from only one subject (219) had low levels of cytotoxicity (5–10% in three independent experiments) directed at targets that were incubated with Rev peptide 780-2 (LLKAVRLIKFLYQSNPPPNF).

DISCUSSION

We have previously shown that the CTL response in an HIV-infected individual to laboratory strain gp160 and RT is dominated

TABLE 2. (*Continued*)

Patient no.	*Env peptide*	*Gag peptide*	*Regulatory peptide*
226		No clear dominant peptide despite gag recognition	nef778-7:aa61-80 EEEEVGFPVTPQVPLRPMTY nef778-8:aa71-90 PQVPLRPMTYKAAVDLSHFL nef778-9:aa81-100 KAAVDLSHFLKEKGGLEGLI
228	env740-2(gp120):aa49-68 VPVWKEATTTLFCASDAKAY	No clear dominant peptide despite gag recognition	nef778-13:aa121-140 PDWQNYTPGPGVRYPLTFGW
230		gag703/8(p17): aa71-85 GSEELRSLYNTVATL gag788.20 (p24): aa323-342 VQNANPDCKTILKALGPAAT	nef778-9:aa81-100 KAAVDLSHFLKEKGGLEGLI
231		gag788.5 (p24): aa173-192 SALSEGATPQDLNTMLNTVG gag788.20 (p24): aa323-342 VQNANPDCKTILKALGPAAT	nef778-9:aa81-100 KAAVDLSHFLKEKGGLEGLI
234	No clear dominant peptide despite env recognition	gag788.14(p24): aa263-282 KRWIILGLNKIVRMYSPTSI	nef778-8:aa71-90 PQVPLRPMTYKAAVDLSHFL nef778-19:aa181-200 EWRFDSRLAFHHVARELHPE nef778-20:aa191-205 HHVARELHPEYFKNC
235	No clear dominant peptide despite env recognition	No clear dominant peptide despite gag recognition	nef778-8:aa71-90 PQVPLRPMTYKAAVDLSHFL nef778-12:aa111-130 LWIYHTQGYFPDWQNYTPGPGV
237	env740-2(gp120):aa49-68 VPVWKEATTTLFCASDAKAY env7E7(gp41):aa844-863 YRAIRHIPRRIRQGLERILL		nef778-12:aa111-130 LWIYHTQGYFPDWQNYTPGPGV nef778-13:aa121-140 PDWQNYTPGPGVRYPLTFGW
307	env701-65(gp41):aa641-655 EIDNYTNTIYTLLEE	No clear dominant peptide despite gag recognition	nef778-12:aa111-130 LWIYHTQGYFPDWQNYTPGPGV
322		gag788.6 (p24): aa183-202 DLNTMLNTVGGHQAAMQMLK gag788.7 (p24): aa193-212 GHQAAMQMLKETINEEAAEW gag788.11 (p24): aa233-252 GSDIAGTTSTLQEQIGWMTN	

by the recognition of a small number of dominant epitopes and several laboratories have shown that T cell recognition of HIV may involve T cells of limited T cell receptor diversity.[6,30–32] This study, which identified CTL epitopes in five HIV gene products (Env, Gag, Nef, Ref, and Tat), covering a large fraction of the HIV proteins, extends our earlier results. This approach has been successful at identifying dominant HIV-1 CTL epitopes in most subjects. It has worked in a reproducible manner because the frequency of HIV-specific CTLs in peripheral blood lymphocytes (PBLs) of infected individuals is high and because the CTL response to the infection in an infected individual is dominated by the recognition of a small number of viral antigens.

In a minority of subjects we were unable to identify any dominant peptides despite recognition of proteins expressed from HIV–vaccinia recombinant-infected autologous cells. This may have been because the recognition was divided among a larger number of peptides than we could detect with the sensitivity of our methods. With minimal specific lysis of a particular gene product by a T cell line of 10–20%, the recognition of even three or four peptides that shared T cell stimulation could have easily been below our sensitivity. Another possible difficulty with our approach is that the HXB.2 strain from which the HIV sequences were derived for generating the vaccinia recombinants differs from the SF2 strain, from which most of the pep-

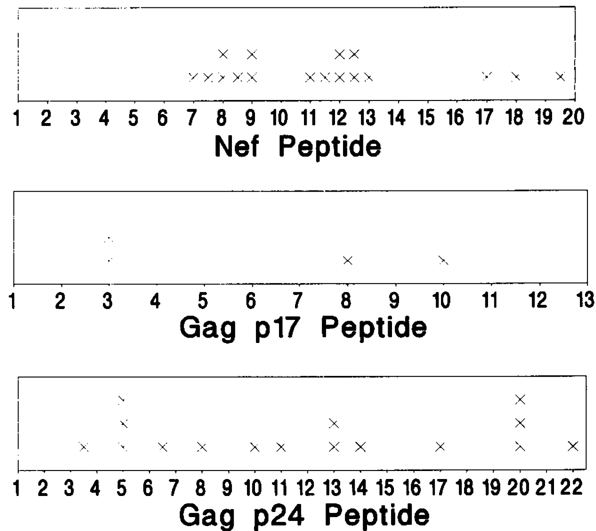

FIG. 3. CTL recognition of peptides in Nef, p17^{gag}, and p24 for the 25 subjects. Recognition of Nef is clustered in the central region. Recognition of Gag is more dispersed but there are some peptides that are recognized by several subjects.

tide sequences were derived and in some cases the 5-aa overlaps of some of the peptide sets may have missed some 8–12 amino acid epitopes.

For this study we have crudely defined epitopes to 15- to 22-mer regions and have not refined the mapping to identify the minimal 8- to 12-amino acid recognized peptides contained within them or identified the MHC-restricting element for their recognition. However, for a subset of the most prevalently recognized gp160 epitopes we have been able to extend the findings presented here to refine the epitopes and define their class I-restricting elements.[17] For the gp160 epitopes recognized by CTLs from four subjects, we have repeated the gp160 epitope mapping after stimulating PBMCs with vPE16-infected autologous targets. Even though Env-specific cytotoxicity increased about fivefold compared to that of the mitogen-stimulated cell line, the epitope mapping was unchanged and no cryptic epitopes were revealed in the cell line with enhanced specific cytotoxicity.[17] Our ability to do this and to enhance peptide-specific cytolysis selectively by exposing mitogen-activated T cell lines to identified 15- to 22-mer recognized peptides supports the accuracy of our methods.[33] Our identification is further supported by the fact that virtually all of the peptides that we identified as CTL epitopes in more than one subject have been previously reported in the literature.

The importance of CTLs that recognize isolate variant epitopes remains unquantitated. Our method and that of all published reports involves looking for epitopes encoded by laboratory strains that differ from the quasispecies infecting an individual. However, it may be that epitopes that are strain specific may be less important, particularly if viral escape from CTL recognition proves significant. The importance of unidentified subdominant epitopes may also change in an individual with disease progression. Because of these considerations, our estimates for the number of viral epitopes recognized provide only a crude lower limit for the diversity of CTL epitopes recognized in infected individuals.

Despite the limitations inherent in our methods, this study is informative about the nature of the CTL response to HIV in a genetically diverse population and has important implications for designing vaccines and immune-based therapies. For CD4 T cell recognition of a number of pathogens, including hepatitis B, tetanus, and malaria, it has been possible to identify a single peptide that is almost universally recognized in a population of diverse MHC class II backgrounds.[34,35] The hope has been that a universal peptide could be defined for HIV CD8 T cell recognition that could be used for incorporation into a vaccine. In lieu of that, it was hoped that defining peptide epitopes recognized in the context of the most prevalent MHC class I alleles, such as *A2*, *A1*, and *A3*, could be used to define a small number of peptides that would be universally recognized.[36] In mapping the immunodominant epitopes recognized by the CTLs of infected individuals, we have found, however, that there is a considerable diversity of HIV epitopes recognized by T cells from infected individuals.[6,17] This result suggests that the class I binding groove for peptides may be more variable than that for class II,[37,38] although other possible explanations could involve differences in MHC class I and II microheterogeneity or in processing of their respective antigens.

The diversity of recognized CTL epitopes is striking in light of the significant overlap in the study population of serologically defined MHC class I expression. Of the 18 individuals for whom peptide epitopes were defined, 8 express *A2*, 7 express *A3*, 6 express *A1* and *A24*, 5 express *B8*, and 4 express *B7*. Seven additional MHC class I alleles (*A11*, *B14*, *B18*, *B35*, *B38*, *B44*, and *B62*) were each expressed by three study subjects. We and others have shown, however, that expressing a particular serologically defined MHC antigen in no way guarantees that the T cells of a subject will recognize any particular epitope.[6,17,19] In fact, we have found that the most common class I alleles infrequently act as restricting elements for CTL recognition of HIV. In a study of the CTL recognition of laboratory strain HIV gp160 by 20 infected subjects (which includes as a subset some of the subjects described here), we did not identify any dominant epitopes that were recognized in the context of common *A1*, *A2*, or *A3* alleles.[17,39] This is despite the presence of a number of peptides encoded by gp160 that contain the anchor motifs required for binding and the identification of peptides that bind with high affinity to these common alleles. CTL recognition of *A2*- and *A3*-restricted gp160 epitopes has, however, developed in heavily vaccinated volunteers. It is possible that these high-affinity epitopes are not efficiently processed or presented by HIV-infected cells but that this inefficiency can be bypassed by intensive immunization. Our findings, that the most common HLA alleles uncommonly act as restricting elements for CTL recognition of HIV and that HLA expression does not predict the recognition of HIV epitopes, together help explain why no 15- to 20-mer peptide in this study was recognized by more than 4 of the 25 (16%) T cell lines studied.

Although there do not appear to be single epitopes recognized by more than a fraction of the population, we and others have shown that there are clusters of multiply restricted epitopes in short regions of gp160, Nef, p17, and RT.[6,17,19,28,40,41] Some of the Gag peptides recognized by multiple individuals in this study have also been reported to contain epitopes restricted by several MHC alleles by other studies and may also

represent CTL epitope clusters.[24] A study of more individuals is required to define Gag clusters since the Gag response is less concentrated than for Nef. Although only a minority of subjects is likely to recognize any one of these clusters, a cocktail composed of 5–10 cluster peptides might be effective at eliciting HIV-specific CTLs in most of the population.

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