

# Patterns of Immunodominance in HIV-1-specific Cytotoxic T Lymphocyte Responses in Two Human Histocompatibility Leukocyte Antigens (HLA)-identical Siblings with HLA-A\*0201 Are Influenced by Epitope Mutation

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## Summary

Primary human immunodeficiency virus (HIV) infection is controlled principally by HIV-specific cytotoxic T lymphocytes (CTL) to a steady-state level of virus load, which strongly influences the ultimate rate of progression to disease. Epitope selection by CTL may be an important determinant of the degree of immune control over the virus. This report describes the CTL responses of two HLA-identical hemophiliac brothers who were exposed to identical batches of Factor VIII and became seropositive within 10 wk of one another. Both have HLA-A\*0201. The CTL responses of the two siblings were very dissimilar, one donor making strong responses to two epitopes within p17 Gag (HLA-A\*0201-restricted SLYNTVATL and HLA-A3-restricted RLRPGGKKK). The sibling responded to neither epitope, but made strong responses to two epitopes presented by HLA-B7. This was not the result of differences in presentation of the epitopes. However, mutations in both immunodominant epitopes of the p17 Gag responder were seen in proviral sequences of the nonresponder. We then documented the CTL responses to two HLA-A\*0201-restricted epitopes, in Gag (SLYNTVATL) and Pol (ILKEPVHGV) in 22 other HIV-infected donors with HLA-A\*0201. The majority (71%) generated responses to the Gag epitope. In the 29% of donors failing to respond to the Gag epitope in standard assays, there was evidence of low frequency memory CTL responses using peptide stimulation of PBMC, and most of these donors also showed mutations in or around the Gag epitope. We concluded that HLA class I genotype determines epitope selection initially but that mutation in immunodominant epitopes can profoundly alter the pattern of CTL response.

CTLs play a central role in the immune response to virus infections (1–4). In HIV infection, CTLs are responsible for the clearance of viremia after primary infection (5–6), and there is strong evidence that they also contribute to prolongation of the disease-free phase of infection (7–10). However, it is not possible to distinguish slow progressors from rapid progressors on the basis of CTL precursor frequency during this asymptomatic phase of infection (9). One explanation is that CTL responses may differ qualitatively. Responses directed at more conserved re-

gions of the virus may be qualitatively superior, because there is less scope for CTL escape mutation to occur without simultaneously damaging the fitness of the virus itself.

Support for qualitative differences in CTL responses derives from studies of HLA associations with rate of progression in HIV infection. HLA class I molecules such as HLA-B27, HLA-B57, and HLA-B51 have been linked with slow progression, while HLA-B8 and HLA-B35 have been associated with rapid development of disease in several studies (11–13). In one study of slow progressors with HLA-B57,

all seven donors tested made the immunodominant response through HLA-B57 rather than any other of their class I molecules (14).

The strong influence of the HLA class I genotype of an individual upon the selection of HIV-specific immunodominant epitopes has been observed for HLA-B27 (10), HLA-B14 (15), and many other examples in other virus infections, for example HLA-A11-restricted Epstein-Barr virus-specific responses (16). In general, patients with a given HLA type react to HIV epitopes in a predictable way (17). Particular MHC molecules may be associated with slow progression because, by chance, the immunodominant epitopes selected are relatively invariant. Switching an individual's immunodominant response away from a variable region of the virus towards a highly conserved region might prove to be a valuable therapeutic option.

It is not known what determines the immunodominance of an individual's CTL response. Possible selective events include specificity of the proteases (18–19), TAP (antigen processing-associated transporter)-dependent transport into the endoplasmic reticulum (20–21), binding affinities of peptides to class I molecules (22), and the stability of peptide-MHC complexes on the cell surface (16, 23). Also, the T cell repertoires may contribute significantly. Some CTL responses to dominant epitopes are oligoclonal, with very similar or even identical TCRs used in different individuals, implying selection among the T cells used by particular epitopes (24–26).

We have studied two HLA-identical hemophiliac siblings within a cohort of HIV-infected donors who have HLA-A\*0201. These brothers first tested seropositive within 10 wk of one another in 1983 and 1984, as a result of exposure to an identical batch of HIV-contaminated Factor VIII concentrate. The CTL responses of these HLA-identical siblings are substantially different, correlating with the presence of striking epitope mutations within the provirus of the nonresponder.

The CTL response of these and 22 other HLA-A\*0201-positive individuals to two well-defined HLA-A\*0201-restricted epitopes, SLYNTVATL (p17 Gag, HIV-1 LAI residues 77–85 [27]) and ILKEPVHGV (Pol, residues 476–484 [28]) has been plotted. The majority of donors generate dominant responses to the Gag epitope, a minority prefer the Pol epitope, and most of this latter group show mutations in or around the Gag epitope.

## Materials and Methods

**Donors.** The 24 HLA-A\*0201-positive adult donors were selected from asymptomatic HIV-infected individuals attending genito-urinary clinics in the Oxford Region, St. Mary's Hospital (London), or the Oxford Haemophilia Centre. No donors had received antiretroviral therapy.

Donors 003 and 023 are HLA-identical hemophiliac brothers, aged 27 and 25, respectively. Both 003 and 023, and also donor 008, were infected between August and December, 1983, after infusion of a particular batch of HIV-contaminated Factor VIII concentrate. These donors were exposed only to this single batch of Factor VIII between August and December, 1983, inclusive,

during which donor 003 received 25,000 U and donor 023 received 27,000 U (the mean usage for a hemophiliac being ~30,000 U per yr; (Spooner, R., personal communication). Donor 023 first became seropositive for HIV in December, 1983 (aged 10 yr), while both 008 and 003 (aged 12 yr) first became seropositive in February, 1984, having been seronegative previously. The brothers 003 and 023, each having a natural factor VIII level of <1%, received very similar doses of Factor VIII. They have lived in the same house throughout their lives; Factor VIII has been exclusively home administered since 1976; all supplies of Factor VIII have been shared; and there has been no batch of Factor VIII of which one brother has received significantly more than the other. The original batches of contaminated Factor VIII unfortunately were unavailable for this study.

Donor 46M first presented in 1994 when her child was diagnosed as HIV infected. The route and duration of infection in 46M are unknown.

**HLA Tissue Typing.** HLA tissue typing was performed by amplification refractory mutation system (ARMS)-PCR using sequence-specific primers (29).

**Peptides.** Peptides were synthesised by Research Genetics, Inc. (Huntsville, AL). The purity of the peptides was determined by HPLC and the identity of the peptide confirmed by mass spectrometry measurement. Peptides were dissolved in DMSO and diluted in RPMI-1640 (Sigma Chem. Co., St. Louis, MO). Overlapping 15–20-mer peptides were supplied by the Medical Research Council AIDS Directed Programme (MRC-ADP, Hertz, UK).

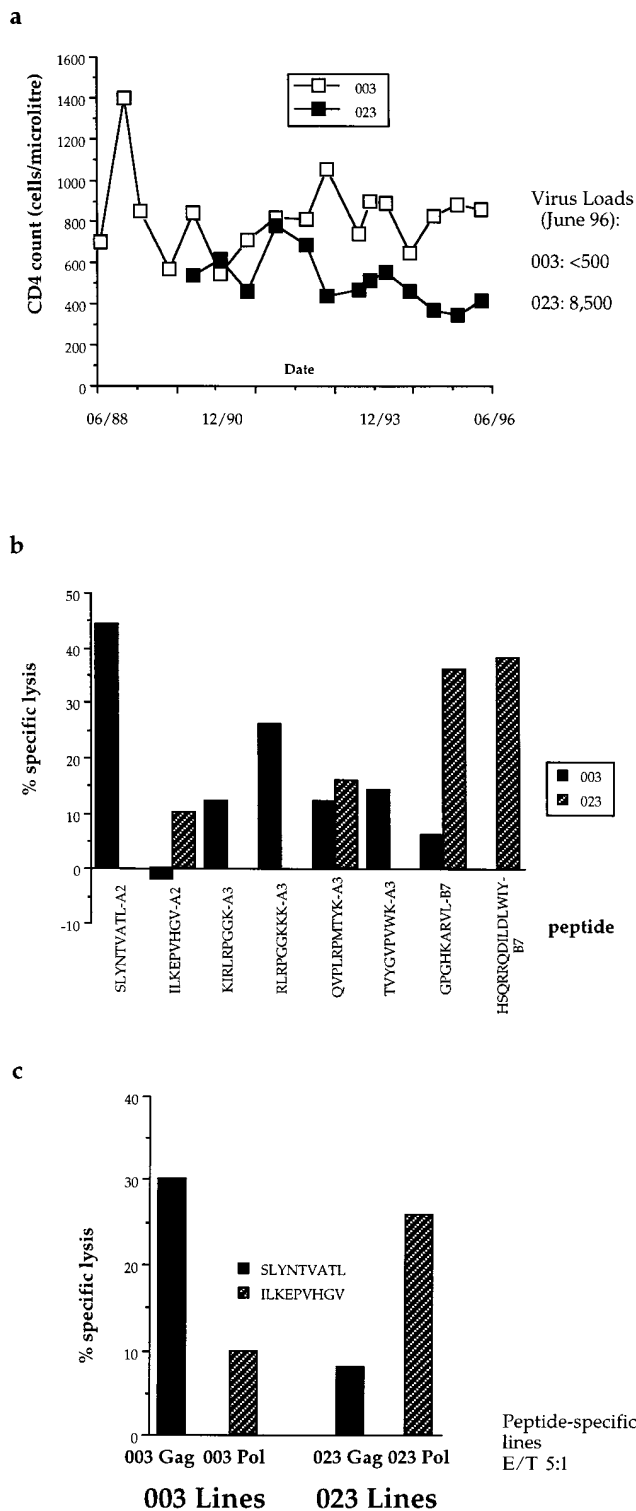
**Recombinant Vaccinia Viruses.** Vaccinia virus recombinants expressing the HIV-1 *pol* and *gp120* genes were provided by the MRC-ADP, the vaccinia expressing the *gag* gene was as described previously (30), that expressing the *nef* gene was provided by Transgene (Strasbourg, France), and the control influenza PB2 vaccinia was kindly provided by Dr. B. Moss (National Institutes of Health, Bethesda, MD).

**Generation of Bulk Cultured CTL and Peptide-specific Lines.** Bulk-cultured CTLs were generated as previously described (30). In brief, one-fifth of PBMC separated from whole blood were added to the other four-fifths after 24 h stimulation with PHA to activate expression of autologous HIV. These cells were cultured in RPMI-1640 supplemented with antibiotics and 10% heat-inactivated FCS (GlobePharm, Esher, UK) (R10) for 7 d, after which 10% Lymphocult T (Biotest) was also added to the medium. Peptide-specific lines were generated by pulsing PBMC with 100  $\mu$ M peptide for 1 h, followed by 3-d culture in R10 with added IL-7 (40 ng/ml). Subsequent media exchanges were performed using R10 containing 10% Lymphocult T. Assays were performed on days 14–24 of culture.

**Identification of Dominant CTL Responses.** Dominant responses were established by assaying bulk CTL against autologous EBV-transformed B-lymphoblastoid cells lines (BCL)<sup>1</sup> infected with a panel of vaccinia recombinants expressing HIV-1 *gp120*, *gag*, *pol*, and *nef* genes. Overlapping peptides (MRC-ADP), 15–20 amino acids in length, were then used to define the region(s) containing recognized epitopes. Additionally, previously defined epitopes presented by matching HLA class I molecules were tested for recognition by bulk CTL.

**CTL Assays.** Vaccinia-infected targets were infected with 3 PFU/cell for 90 min, washed, and incubated in R10 overnight. In peptide-based assays, targets were pulsed with peptide for 1 h,

<sup>1</sup>Abbreviations used in this paper: ARMS, amplification refractory mutation system; BCL, B-lymphoblastoid cell lines; TAP, transporter associated with antigen processing.



**Figure 1.** (a) Serial CD4 counts and latest viral loads in donors 003 and 023. (b) Pattern of recognition of CTL epitopes in bulk cultured lymphocytes from donors 003 and 023. E/T ratio, 50:1. Only peptides recognized by lymphocytes from 003 or 023 are shown. HLA-A\*0201-restricted epitopes, SLYNTVATL and ILKEPVHGV; HLA-A3-restricted epitopes, KIRLRPGGK, QVPLRPMTYK, TVYGVVWVK (reference 17), and RLRPGGKKK; HLA-B7-restricted epitopes, GPGHKARVL and HSQRQDILDLDWIY (Goulder, P.J.R., unpublished data). Lysis of targets pulsed with no peptide subtracted to calculate percent specific lysis. Assay

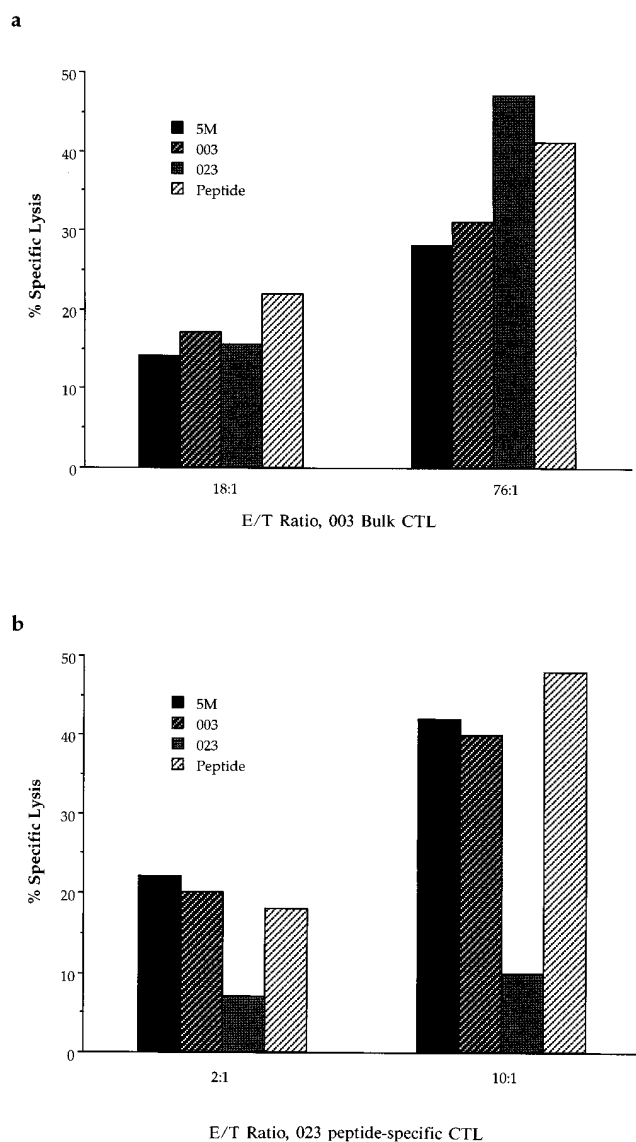
washed, and aliquots of  $5 \times 10^3$ /well in 100  $\mu$ l were used in duplicates. Targets were labeled with  $^{51}\text{Cr}$  and washed a total of three times before use in the assay. Effectors were added to each well in 100  $\mu$ l and incubated with targets for 4–6 h. Percent lysis was calculated from the formula:  $100 \times (E - M)/(T - M)$ , where E was the chromium release from 20  $\mu$ l supernatant of wells containing targets and effectors, M the release from wells containing targets and medium only, and T the release from wells containing targets and 5% Triton X-100. Where the chromium release from duplicate wells differed by >15% from the mean, the data were discarded. Spontaneous release (M/T) was always <30%. Percent of specific lysis was calculated by subtracting percent lysis of targets pulsed with no peptide or infected with the control flu-vaccinia from percent lysis of targets pulsed with peptide or infected with HIV-1 protein-vaccinia, respectively.

**Amplification, Cloning and Sequencing of Proviral DNA.** Full-length p17 gag was amplified from proviral DNA isolated from PHA-activated PBMC using nested PCR. The primers used were: 5'-GTTCTAGGTGATATGG-3', 5'-ACTAGCGGAGGCTAG-3' for the primary reaction; and 5'-AATTCCATGGGTGCGAGAGCGT-3' and 5'-CAGTTCTAGATCTAGTAATTTGGGCTGACC-3' for the secondary reaction. PCR products were cloned into the T vector system (Amersham) and epitopes sequenced with insert-specific primers using the Sequenase Version 2.0 protocol (USB, Cleveland, OH).

## Results

**CTL Responses, CD4 Count, and Virus Load in Two HLA-identical Siblings Infected at the Same Time.** The HLA class I and class II tissue type of the HLA-identical siblings, donors 003 and 023, was the following: HLA-A\*0201, -A\*03 -B\*07, -B\*51 -Bw\*4, -Bw6 -Cw\*7, -Cw14 -DR\*1, -DR\*8 -DQ\*4, -DQ\*5. The respective CD4 counts and virus load are shown in Fig. 1 a. The CTL response profile was quite different in the two brothers (Fig. 1 b, Table 1). CTL from donor 003 recognized the HLA-A\*0201-restricted peptide SLYNTVATL as the immunodominant response, but two HLA-A3-restricted epitopes within p17 Gag, one in Nef, and one in Env were also seen by CTL from this donor. The dominant responses identified in donor 023 were to two HLA-B7-restricted epitopes not previously described (Fig. 1 b), one in p24 Gag, and one in Nef, neither of which was recognized by CTL from donor 003. A relatively weak response to the HLA-A\*0201 epitope in Pol ILKEPVHGV was identified in bulk-cultured lymphocytes from donor 023. This peptide was not seen in the bulk response by 003, although it could be seen as a weak response when PBMC were stimulated directly by the peptide (Fig. 1 c). Conversely, no responses were observed in donor 023 to the HLA-A\*0201- and HLA-A3-restricted p17 Gag epitopes, the strongest responses observed in bulk CTL from donor 003 (Fig. 1 b). This pattern of Gag-specific CTL responses observed in donors 003 and 023 has been stable since 1986 (31).

timepoint, January, 1996; very similar responses were observed from timepoint July, 1996. (c) Peptide stimulation of PBMC allows detection of Pol-specific CTL in donor 003 and Gag-specific CTL in donor 023. Assay timepoint, June, 1994; very similar responses were observed from the December, 1994 timepoint.



**Figure 2.** (a) Bulk cultured CTL from donor 003 recognized Gag-vaccinia-infected BCL targets from donor 023 (who made no HLA-A2- or HLA-A3-restricted Gag response; HLA-identical) and from donor 5M (a Gag responder; HLA matched through HLA-A\*0201 and HLA-B51). E/T ratio, 18:1 and 76:1 as shown. Recognition of targets pulsed with 10  $\mu$ m SLYNTVATL also is shown. Lysis of targets pulsed with no peptide or infected with control PB2-vaccinia subtracted to calculate percent specific lysis. In separate assays, BCL from Pol responders 023, 065, and 008 similarly presented the SLYNTVATL epitope to Gag-specific effectors from donors 102, 077, and 868 (data not shown). (b) HLA-A\*0201-restricted ILKEPVHGV (Pol)-specific line from donor 023 recognized Pol-vaccinia-infected BCL targets from two Gag responders, 003 (HLA-identical) and 5M (matched through HLA-A\*0201 and HLA-B51). Recognition of BCL targets pulsed with 10  $\mu$ m ILKEPVHGV also is shown. In separate assays, BCL from Gag responders 46M and 868 similarly presented the ILKEPVHGV epitope to Pol-specific effectors from donors 241, 46M, and 868 (data not shown).

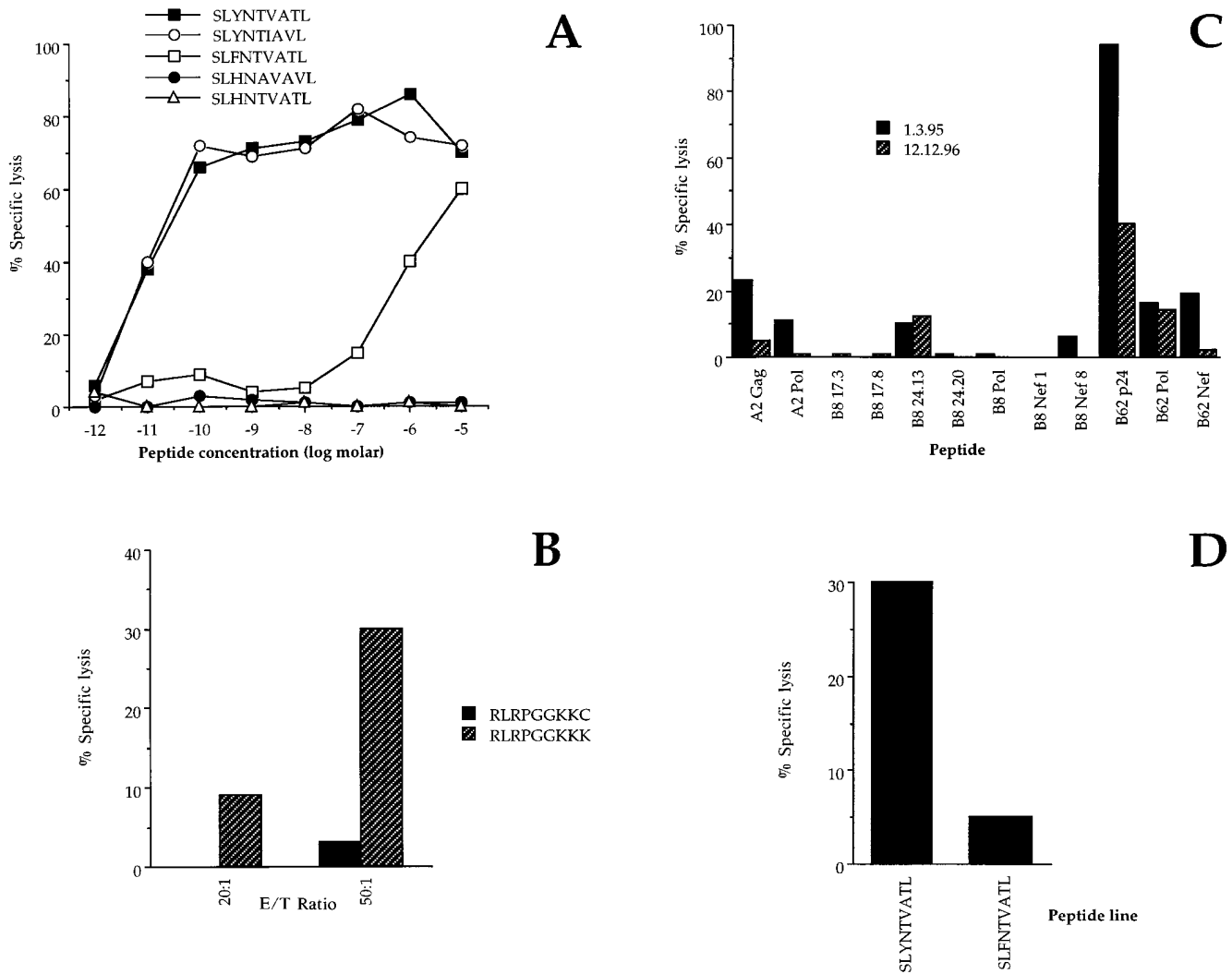
*Gag-Vaccinia-infected Targets from Donor 023 Efficiently Present the SLYNTVATL Gag Epitope to 003 CTL, and Pol-Vaccinia-infected Targets from Donor 003 Efficiently Present the ILKEPVHGV Pol Peptide to 023 CTL.* One potential explanation for the relative failure of donors to generate a re-

**Table 1.** HLA-A\*0201-positive Donors Tested

Donor	Gag response	Pol Response	HLA-A and HLA-B type	
<b>Gag Responders</b>				
1	003	40	3	A2/3 B7/51
2	031	28	14	A1/2 B51/57*
3	077	22	10	A2/3 B41/49
4	079	10	3	A2/32 B7/27
5	102	22	4	A2/- B7/57
6	104	28	4	A2/11 B7/62
7	324	12	6	A2/11 B7/60
8	1M	50	2	A1/2 B7/57
9	2M	29	3	A2/6802 B42/51
10	5M	52	2	A2/32 B7/57
11	22M	18	6	A2/6802 B41/72
12	25M	12	5	A2/3 B7/60
13	46M	23	11	A1/2 B8/62
14	48M	34	ND	A2/29 B35/44*
15	114	23	12	A2/3 B35/-*
16	868	38	23	A2/24 B27/35
<b>Pol Responders</b>				
1	023	3	10	A2/3 B7/51
2	008	2	21	A2/6801 B8/65
3	241	6	30	A2/26 B39/62
4	065	4	38	A1/2 B8/44
5	824	0	11	A1/2 B44/57
<b>Equal Gag and Pol/Nonresponders</b>				
1	069	21	21	A2/31 B60/62
2	606	3	1	A2/- B62/65
3	SC7	2	0	A1/2 B7/44*

HLA class I -A and -B types shown. Gag responders: bulk CTL at E/T 50:1 recognizes the Gag epitope SLYNTVATL, with percent specific lysis >10% (after subtraction of lysis of targets pulsed with no peptide). Specific killing of ILKEPVHGV (Pol) pulsed targets (10  $\mu$ m) is shown. Pol responders: dominant HLA-A\*0201-restricted response is to the Pol epitope. Equal responders: no preference for Gag or Pol epitope. The bulk responses of most of the donors was assayed on several (2-6) occasions; those asterisked were assayed on a single occasion only.

sponse to a particular epitope is that autologous antigen-presenting cells may fail to process it. The possibility of processing polymorphism being associated with a particular response was not the explanation in donors 003 and 023, who are HLA class I and class II identical. Using Gag-vaccinia-infected BCL from 023 and from an HLA-A\*0201-matched donor (5M) as targets, the Gag peptide SLYNTVATL was processed and presented as efficiently to 003 bulk CTL as by autologous BCL (Fig. 2 a). Likewise, BCL from Gag responders were able to process and present the



**Figure 3.** (a) Recognition of SLYNTVATL variants by a SLYNTVATL-specific line from donor 868; E/T 3:1. Lysis of targets pulsed with no peptide subtracted to calculate percent specific lysis. This assay was repeated on three occasions with the same pattern of peptide recognition (data not shown). Bulk-cultured lymphocytes from donor 003 also showed no recognition of the SLHNAVAVL variant (0% specific lysis) compared with recognition of SLYNTVATL (45% specific lysis) (data not shown). (b) Recognition of the HLA-A3-restricted peptide epitope RLRPGGKKK by bulk-cultured CTL from donor 003, and the variant RLRPGGKKC (encoded by proviral DNA within donor 023); E/T ratios as shown. Peptide concentrations, 50  $\mu$ M. Peptide titration assays were also performed using a peptide-specific line from donor 003: the variant peptide RLRPGGKKC was not recognized at peptide concentrations of below 50  $\mu$ M, whereas the index peptide was recognized above peptide concentrations of 5 nM (data not shown). (c) Recognition of defined HLA-A\*0201-, HLA-B8-, and HLA-B62-restricted CTL epitopes (references 17 and 38) by bulk-cultured CTL from donor 46M (HLA class I -A and -B tissue type: HLA-A1/2 -B8/62) at two timepoints: March, 1995, and December, 1996. Bulk CTL cultured for 15–16 d in each case before assay. E/T ratio, 100:1. Peptide concentrations: 10  $\mu$ M. A2 Gag, SLYNTVATL. A2 Pol, ILKEPVHGV. B8 17.3, GGKKKYKL. B8 17.8, ELRSLYNTV. B8 24.13, EIYKRWII. B8 24.20, DCKTILKAL. B8 Pol, GPKVKQWPL. B8 Nef 1, WPTVRERM. B8 Nef 8, FLKEKGGL. B62 p24, GLNKIVRMY. B62 Pol, ILKEPVHGVY. B62 Nef, TQGYFPDWQNY. (d) SLYNTVATL-specific killing is not detectable in peptide-specific lines stimulated using SLFNTVATL, but is generated using the index peptide to stimulate PBMC from donor 46M. E/T ratios: SLFNTVATL line, 14:1; SLYNTVATL line, 6:1. Lysis of targets pulsed with no peptide subtracted to calculate percent specific lysis. Assay performed at day 16 of culture; the same result was observed at day 23 of culture (data not shown).

Pol peptide very efficiently (Fig. 2 b). Gag-vaccinia-infected BCL from other Gag nonresponders (see below) similarly were able to present SLYNTVATL efficiently to 003 CTL (data not shown).

*Analysis of p17 Gag Sequences from Proviral DNA in Donors 003 and 023.* Provirus DNA from donor 003 does not differ significantly from the consensus B clade (wild-type) Gag sequence, and in particular, no deviation from the wild-

type sequence encoding the two immunodominant epitopes SLYNTVATL (HLA-A\*0201) and RLRPGGKKK (HLA-A3; our unpublished data) was seen in more than 40 clones (Table 2). In contrast, proviral DNA from donor 023 differs strikingly from the wild-type sequence. The proviral sequences from 023 encoding the two epitopes best recognized by CTL from 003 were significantly altered, encoding the peptides SLHNAVAVL and RLRPGGKKC in

**Table 2.** Deduced Amino Acid Sequence around SLYNTVATL Epitope from Proviral DNA Sequence in Responders and Nonresponders

Donor	HLA class I A and B type	Timepoint	Deduced amino acid sequence	% Proviral DNA clones			
SLYNTVATL Responders							
003	A2/3 B7/51	07/87	GSEELRSLYNTVATLYCVHQR	100%	<i>n</i> = 8		
			06/95	GSEELRSLYNTVATLYCVHQR	82%	<i>n</i> = 13	
			GSEERKSLYNTVATLYCVHQR	6%	<i>n</i> = 1		
			GSEELRSLNTVATLYCVHQR	6%	<i>n</i> = 1		
			GSEELKSLYNTVATLYCVHQR	6%	<i>n</i> = 1		
		12/95	GSEELRSLYNTVATLYCVHQR	100%	<i>n</i> = 16		
		06/96	GSEELRSLYNTVATLYCVHQR	100%	<i>n</i> = 5		
		077	A2/3 B41/49	12/94	GSEELRSLYNTVATLYCVHQR	100%	<i>n</i> = 3
				1M	A2/1 B7/57	08/96	GSEERKSLYNTVATLYCVHQR
		5M	A2/32 B7/57	10/93	GSEELRSLYNTIATLYCVHQR	90%	<i>n</i> = 28
GSEELRSLYNTVAVLYCVHQR	10%				<i>n</i> = 3		
868	A2/24 B27/35	11/95	GSEEVRSLYNTVATLYCVHQR	100%	<i>n</i> = 4		
		10/95	GSEELRSLYNTVATLYCVHQR	73%	<i>n</i> = 8		
			GSEELRSLYNTIATLYCVHQR	9%	<i>n</i> = 1		
			GSEELKSLYNTIATLYCVHQR	9%	<i>n</i> = 1		
		04/96	GSEERKSLYNTVATLYCVHQR	9%	<i>n</i> = 1		
		08/96	GSEELRSLYNTIATLYCVHQR	92%	<i>n</i> = 11		
			GSEELKSLYNTIATLYCVHQR	8%	<i>n</i> = 1		
		GSEELKSLYNTVATLYCVHQR	100%	<i>n</i> = 5			
SLYNTVATL Nonresponders							
023	A2/3 B7/51	07/87	GSEKLKSLHNTVATLYCVHQR	100%	<i>n</i> = 3		
			06/95	GSEELQSLHNAVAVLYCVHQR	70%	<i>n</i> = 7	
		12/95	GSEELKSLYNTVATLYCVHQR	10%	<i>n</i> = 1		
			GSEELKSLYNTIATLYCVHQR	10%	<i>n</i> = 1		
			GSEELKSLSNTIATLYCVHQR	10%	<i>n</i> = 1		
			GSEELQSLHNAVAVLYCVHQR	100%	<i>n</i> = 10		
07/96	GSEELQSLHNAVAVLYCVHQR	100%	<i>n</i> = 16				
008	A2/68 B8/65	04/91	GSEEFRLSFLNTVATLYCVHQR	50%	<i>n</i> = 3		

Continued

100% of sequences cloned from the most recent timepoint. No response to these peptides or the wild-type peptides was seen in bulk CTL from donor 023 (see Fig. 1 *b*; data not shown) and no response was made to the variant peptides by bulk CTL from donor 003 (Fig. 3 *b*; data not shown). The distinctive epitope changes (His for Tyr at position 3 in the SLYNTVATL epitope) observed in proviral DNA from donor 023 have been stably present at least since the earliest timepoint sequenced, a period of almost a decade, and similarly the wild-type sequence has been stable within donor 003 for this duration (Table 2). Thus, the two immunodominant responses in 003 may be absent in 023 because both epitopes have mutated to non-recognized forms.

*SLYNTVATL-specific CTL Dominate the HLA-A\*0201-restricted in 24 HIV-infected Donors.* To determine which of these responses was typical of HIV-positive donors with HLA-A\*0201, we determined the HLA-A\*0201-restricted

CTL responses in 22 further donors, using autologous virus-infected activated T cells to stimulate the responses. One hemophiliac donor, 008, was infected by the same batch of contaminated Factor VIII as 003 and 023. One donor made no detectable CTL response (SC7), and in two donors there was an equal response to the Gag and Pol epitopes (069, responses to both peptides; and 606, responses to neither). The response in donor 48M to the Pol peptide was not tested. Bulk CTL from 15 of the other 20 donors preferentially responded to the Gag epitope, while 5 (including 023 and 008) preferentially responded to the Pol epitope.

*Provirus Sequences from Gag Nonresponders Encoding the Gag Epitope Show a Greater Divergence from the Consensus Sequence.* Analysis of the p17 gag sequences within Gag responders shows that there is little divergence away from the wild-type sequence (Table 2). In contrast, there was greater variation in this region in sequences from Gag nonre-

**Table 2.** *Continued*

Donor	HLA class I A and B type	Timepoint	Deduced amino acid sequence	% Proviral DNA clones	
065	A2/1 B8/44	04/92	GSEEF <u>RS</u> SLFNTVATLYCVHQR	17%	<i>n</i> = 1
			GSEEF <u>RS</u> LLNTVATLYCVHQR	17%	<i>n</i> = 1
			GSEEF <u>RS</u> LSNTVATLYCVHQR	17%	<i>n</i> = 1
			GSEEL <u>K</u> SLFNTVAVLYCVHQR	38%	<i>n</i> = 8
			GSEEL <u>R</u> SLFNTVAVLYCVHQR	19%	<i>n</i> = 4
			GSEEL <u>K</u> SLFNTIAVLYCVHQR	10%	<i>n</i> = 2
			GSEEF <u>RS</u> SLFNTVATLYCVHQR	5%	<i>n</i> = 1
			GSEEL <u>K</u> SLFNTVATLYCVHQR	14%	<i>n</i> = 3
			GSEEL <u>R</u> SLYNTIAVLYCVHQR	14%	<i>n</i> = 3
065	A2/1 B8/44	02/93	GSEEL <u>K</u> SLYNTVATLYCVHQR	100%	<i>n</i> = 28
		08/94	GSEEL <u>K</u> SLYNTVATLYCVHQR	100%	<i>n</i> = 1
		05/96	GSEEL <u>K</u> SLYNTVATLYCVHQR	100%	<i>n</i> = 2
241	A2/26 B39/62	02/93	GSEEL <u>K</u> SLYNTIATLYCVHQQ	100%	<i>n</i> = 6
			606	A2/- B62/65	10/94
606	A2/- B62/65	10/94	GSEEL <u>K</u> SLFNTVAVLYCVHQR	25%	<i>n</i> = 1
			SLYNTVATL Responder Switched to Nonresponder		
46M	A2/1 B8/62	09/94	GSEEL <u>R</u> SLYNTVATLYCVHQR	33%	<i>n</i> = 6
			GSEEL <u>K</u> SLYNTVATLYCVHQR	17%	<i>n</i> = 3
			GSEEL <u>R</u> SLFNTVATLYCVHQR	11%	<i>n</i> = 2
		06/95	GSEEL <u>K</u> SLFNTVATLYCVHQR	39%	<i>n</i> = 7
			GSEEL <u>K</u> SLFNTVATLYCVHQR	100%	<i>n</i> = 2
			04/96	GTEEL <u>R</u> SLFNTVATLYCVHQR	100%

Proviral DNA sequences and the encoded amino acid sequences at the region of the Gag epitope for five Gag responders, five nonresponders, and one donor whose Gag response disappeared between the first timepoint and the last; showing the timepoint (mo/yr), amino acid sequence (SLYNTVATL epitope underlined, with six amino acids either side of the epitope shown; differences from HIV<sub>LAI</sub> sequence shown in bold); number of clones sequenced, and proportion encoding a particular amino acid sequence.

sponders. In donors 008 and 606 (Gag nonresponders), the encoded Gag peptides were not well recognized by peptide-specific CTL, whereas variation transiently observed in the Gag responder 868 did not affect CTL recognition (Fig. 3 a). Although mutations within the epitope and within amino acids flanking the epitope were observed more frequently in Gag nonresponders, some nonresponders (065 and 241) carried proviral sequences encoding changes within the epitope that did not alter CTL recognition, and some Gag responders carry sequences encoding changes flanking the epitope (1M, 868).

*Switch from Gag Responder to Nonresponder in Donor 46M Coincided with Fixation of a Sequence Change Resulting in Failure to Recognize the Epitope.* When bulk cultured CTL from donor 46M were first assayed in 1994, a clear response to the SLYNTVATL Gag epitope was observed. Subsequently, this response has disappeared (Fig. 3 d), while other responses remain readily detectable. In 1994, 50% of autologous provirus sequences encoded the index peptide; 2 yr later, 100% of sequences encode the variant SLFNTVATL (Table 2). This Tyr→Phe change at position 3 in the epitope

results in poor CTL recognition (Fig. 3 a). In all Gag nonresponders tested so far (023, 241, 065, 606, and 46M; timepoint 12/96) stimulation of PBMC with the SLYNTVATL peptide allowed detection of SLYNTVATL-specific CTL (specific lysis at E/T ratio of 5:1 ranging from 9 to 40%; Fig. 3 c; data not shown), but stimulation of 46M PBMC with the SLFNTVATL variant did not result in the generation of any peptide-specific CTL (Fig. 3 d).

## Discussion

CTLs play a central role in the immune response to virus infections, as well as to other intracellular pathogens and to tumor cells. The importance of CTL at all stages of HIV infection is increasingly apparent. There is also growing evidence that there are qualitative differences in the responses generated by infected individuals, and that these may influence the long-term control of the infection and, ultimately, the prognosis. The determinants of immunodominance of MHC class I-restricted CTL responses are of considerable relevance to disease progression.

We have studied the HIV-specific CTL responses directed through the commonest HLA class I molecule found in caucasoid and many other populations, HLA-A\*0201 (32). The majority of donors recognise the Gag epitope SLYNTVATL predominantly, and a minority, the Pol epitope, ILKEPVHGV. Because the response is not universally directed towards one epitope rather than the other, the explanation cannot lie solely in differences of affinity of the peptides to HLA-A\*0201. In fact, our binding studies show that the Pol peptide binds significantly better than the Gag peptide (data not shown), and other studies have similarly shown very strong binding of the Pol peptide to HLA-A2 (33). This is consistent with what is known of the peptide-binding motif for HLA-A\*0201, where the preferred anchor residues are leucine at position 2 and valine at position 9 (COOH-terminal anchor, PC). Of 16 HLA-A\*0201-restricted CTL epitopes listed (34), 12 had valine at PC, 2 had leucine. A previous study comparing the relative binding affinities of polyalanine substituted amino acids to HLA-A\*0201 showed that the substitution of valine for leucine at PC resulted in a 10-fold increase in relative binding (35).

The predominance of CTL responses to the Gag epitope is in better agreement with data estimating the abundance of each peptide on the cell surface, where >30 times as many SLYNTVATL molecules were present on the surface of stably infected Jurkat-A2 cells as ILKEPVHGV molecules (27). We investigated the possibility that HIV-infected cells from Gag responders might be presenting the SLYNTVATL peptide more efficiently than the Pol peptide, but experiments using Gag-vaccinia-infected BCL did not support this (Fig. 2 a; data not shown). Similarly, BCL from Gag responders, when infected with Pol-vaccinia, presented the Pol peptide at least as well as Pol-vaccinia-infected BCL from a Pol responder (Fig. 2 b).

The observation has often been made that HIV-specific CTL epitopes tend to cluster in immunodominant regions of proteins (17). Because of the report of HLA-B8 and HLA-B\*2702 MHC class I molecules competing for the presentation of the overlapping influenza-specific epitopes ELRSRYWAI and LRSRYWAI (36), the possibility was raised that this phenomenon might influence immunodominance of HIV-specific CTL responses similarly. Whereas donor 065 generated no response to the Gag epitope SLYNTVATL, a strong response was made to the HLA-B8-restricted overlapping epitope ELRSYNTV (Goulder, P.J.R., S.W. Reid, D.A. Price, A.J. McMichael, R.E. Phillips, and E.Y. Jones, manuscript submitted for publication), and a weaker response to the HLA-A1-restricted epitope, GSEELRSLY (our unpublished data). Although Gag-vaccinia-infected HLA-A\*0201/B8 targets appeared to express the SLYNTVATL epitope adequately (data not shown), we cannot exclude that competition between MHC molecules for overlapping epitope peptides could influence the development of immunodominance of the response, favoring a predominant response to the Pol epitope in this donor.

Donors 003 and 023 are two HLA-identical brothers who were infected by a virus at the same time, probably the same quasispecies of virus, at a very similar dose. Nei-

ther donor has progressed to disease over 12 yr of infection. Donor 003 has a virus load below the level of detection (<500 RNA copies/ml), and that of donor 023 is low (8,500 RNA copies/ml plasma); neither has received anti-retroviral therapy. The CTL responses are quite different, which demonstrates that, in this instance, the pattern of response is not determined solely by the HLA genotype, or by TAP polymorphism or by proteasome-related processing effects.

It is difficult to demonstrate unequivocally that viral isolates from the two individuals have derived from a single original virus, because selection acting early on in the course of infection may have resulted in dramatically divergent sequences. Unfortunately, we do not have access to the contaminated batch of Factor VIII believed to have infected these two brothers, and the earliest sequence available for analysis is 3 yr after infection. Comparison of p17 gag sequences would show major dissimilarities between 003 and 023 sequences, because (as argued below) selection pressure exerted by CTL early on in infection has resulted in epitope variation and switching of immunodominant responses to different epitopes in donor 023. Neighboring sequences outside epitopes might be expected to show compensatory changes, increasing the interindividual sequence divergence further. Comparison of other regions (such as *env*) of the genome might be equally misleading because of different selective pressures acting on the viruses in the two brothers.

The clear epidemiological evidence that these brothers were infected with the same original virus is the detailed documentation that throughout life they have shared identical supplies of Factor VIII, they were exposed at the same time exclusively to one particular batch of Factor VIII, and shortly afterwards both brothers seroconverted. Donors 003 and 023 are now 27 and 25 yr of age, respectively, and first became seropositive within 10 wk of one another (December, 1983 and February, 1984) when aged 12 and 10 yr, having been exposed by home-administered treatment exclusively to the same batch of non-heat-treated Factor VIII between August and December, 1983.

The study by Tsomides et al. (27) implies that an excess of the Gag SLYNTVATL epitope compared with the Pol ILKEPVHGV epitope is presented by infected CD4<sup>+</sup> cells in both donor 003 and donor 023 (27). The remarkable difference between these two donors is in the provirus sequence encoding the Gag epitope. The peptide encoded by 100% of provirus from donor 023, SLHNAVAVL, is not recognized by autologous bulk CTL or Gag-specific CTL lines (Fig. 3 a). Equally striking is the sequence change observed in provirus from donor 023 encoding the HLA-A3-restricted epitope, RLRPGGKKK, the second of two dominant responses in donor 003 (Fig. 1 b). In this case, the PC anchor lysine residue is switched to a cysteine, a change resulting in abrogation of recognition by CTL from donor 003 (Fig. 3 b). (No epitope peptide has been described with a cysteine at the COOH terminus [34], so this peptide may not even be processed or transported.) This combination of mutations is unique for each epitope, when compared with 32 B clade Gag amino acid sequences in the database (38). It is



also noteworthy that the unusual Tyr→His sequence change has been stable for at least 9 yr.

Are the unique sequence changes observed in the provirus of donor 023 and the unusual preference in the CTL response of this donor for the Pol epitope causally connected? In another hemophiliac donor, 008, also probably infected by the same viral quasispecies as donors 003 and 023, provirus also encodes an epitope that is not recognized by Gag-specific CTL, and this donor, too, is unusual in being a Pol responder. Together with the PC anchor sequence change observed within the HLA-A3-restricted epitope RLRPGGKKK in donor 023 provirus, these data are suggestive of CTL escape occurring early on in infection and strongly influencing the subsequent stable pattern of CTL response observed. In donor 023, two mutations together might permit escape from both CTL epitopes, whereas in 003 this does not occur, perhaps because of the presence of other CTL responses.

The disappearance of a bulk response to SLYNTVATL in donor 46M in association with the increased frequency to fixation of the F3 variant is suggestive that this pattern of events may have occurred in other Gag nonresponders. In support of this is the finding that SLYNTVATL-specific CTL could be detected using the sensitive method of peptide stimulation of PBMC in all Gag nonresponders tested, implying the presence of memory CTL. Use of SLYNTVATL-HLA-A\*0201 complexes (39) has shown that some Gag nonresponders nonetheless have a relatively high frequency of SLYNTVATL-specific effectors within PBMC, which implies that these Gag nonresponders have previously generated SLYNTVATL-specific responses that are no longer active.

A previous study described two donors with HLA-A11 and three other donors with HLA-B18, who made CTL responses to Nef-specific epitopes, provided that autologous virus sequences did not show mutations that abrogated CTL recognition (40). The data we describe show that such epitope variation is not necessary for a failure of a response towards the epitope to be observed; but often this is the case, and may be the result of multiple early escape muta-

tions, occurring within 3 yr of seroconversion. Studies of CD8<sup>+</sup> TCR V $\beta$  usage after seroconversion have illustrated the spectacular V $\beta$ -specific expansions and deletions that occur rapidly at this time (41). The swift emergence of CTL escape mutations at seroconversion has also been well described (42–43). The precise relationship of these dramatic fluctuations to escape variation within the cognate epitope has not been determined.

It is clear from these data that early escape mutation within the normally immunodominant epitope SLYNTVATL is not the only factor determining the pattern of an individual CTL response. It is possible, as in the Pol responder 065 (whose sequence carries no mutations within the epitope), that competition between class I molecules for overlapping epitopes may have an influence. In this study, we have not investigated the role of flanking mutations, which occurred universally in the Gag nonresponder group (including 065) and relatively infrequently in the Gag responder group (Table 2). The clustering of nonsynonymous mutations at sites flanking HIV-specific CTL epitopes has been noted previously (40, 44), and flanking changes have been demonstrated to affect presentation in some systems (19, 45).

In summary, the immunodominance of CTL responses in two HLA-identical siblings, probably infected by the same quasispecies of virus and at the same time, is not determined by HLA genotype or processing differences alone. The CTL responses in HLA-A\*0201 seropositive donors to two well-defined HLA-A\*0201-restricted epitopes is likely to be determined primarily by the high abundance on the cell surface of the Gag epitope relative to the Pol epitope. In the minority of individuals who fail to respond to the Gag epitope, mutation within the Gag epitope occurring early in infection that abrogates CTL recognition may influence the pattern of the CTL response away from the Gag epitope. In addition, variation within flanking regions, reducing the presentation of the Gag epitope, or competition by other HLA class I molecules for epitopes overlapping with the Gag epitope, may also determine the pattern of the CTL response.

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