Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS

PHILIP J.R. GOULDER[1], RODNEY E. PHILLIPS[1], ROBERT A. COLBERT[2], STEPHEN MCADAM[1], GRAHAM OGG[1], MARTIN A. NOWAK[3], PAUL GIANGRANDE[4], GRAZ LUZZI[5], BARBARA MORGAN[6], ANNE EDWARDS[6], ANDREW J. MCMICHAEL[1] & SARAH ROWLAND-JONES[1]

[1]*Nuffield Department of Clinical Medicine and Institute of Molecular Medicine, University of Oxford, Oxford OX3 9DU, UK*
[2]*The William S. Rowe Division of Rheumatology, Children's Hospital Medical Center, 3333 Burnet Ave., Cincinnati, Ohio 45229-3039, USA*
[3]*Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, UK*
[4]*Oxford Haemophilia Centre, Churchill Hospital, Oxford OX3 7LJ, UK*
[5]*Wycombe General Hospital, South Buckinghamshire NHS Trust, Queen Alexandra Road, High Wycombe Bucks, HP11 2TT, UK*
[6]*Radcliffe Infirmary, Woodstock Road, Oxford OX2 6HE, UK*

The precise role played by HIV-specific cytotoxic T lymphocytes (CTL) in HIV infection remains controversial. Despite strong CTL responses being generated during the asymptomatic phase, the virus persists and AIDS ultimately develops. It has been argued that the virus is so variable, and the virus turnover so great that escape from CTL recognition would occur continually, but so far there is limited evidence for CTL escape. The opposing argument is that evidence for CTL escape is present but hard to find because multiple anti-HIV immune responses are acting simultaneously during the asymptomatic phase of infection. We describe six donors who make a strong CTL response to an immunodominant HLA-B27-restricted epitope. In the two donors who progressed to AIDS, CTL escape to fixation by the same mutation was observed, but only after 9–12 years of epitope stability. CTL escape may play an important role in the pathogenesis of HIV infection.

There is increasing evidence that cytotoxic T lymphocytes (CTLs) play a central role in the immune response to HIV. They are activated *in vivo* to high levels at the same time as the initial clearance of the primary viremia[1-3]. It is also clear that they are important in the maintenance of the asymptomatic phase of infection before the development of the acquired immune deficiency syndrome (AIDS)[4-6]. During this phase, they are present at relatively high levels[7] and can often be demonstrated *ex vivo* without a requirement for *in vitro* stimulation and expansion.

Mutation and consequent virus variation is a characteristic feature of human immunodeficiency virus (HIV) infection. Variation in the epitopes recognized by CTLs and fluctuation in the frequency of mutant viruses in the quasispecies have been described for donors who make CTL responses through human leukocyte antigens HLA-B8 (ref. 8, 9), HLA-A11 and HLA-B18 (ref. 10) and HLA-A3 (ref. 11). It has been argued that this represents escape from CTL responses. However, the frequent coexistence of CTL responses to more than one epitope has made interpretation complex[9]. It is possible that polyvalent CTL responses to multiple epitopes are the result of a series of CTL escapes to a succession of immunodominant epitopes[9]; it is also possible that small decreases in efficiency of the total CTL response could give mutant viruses an advantage even in the face of CTL responses to several epitopes. However, preferential selection of mutant epitopes is not always seen in asymptomatic seropositive donors[12,13]. Thus it is possible that epitope mutation does not always play a major role in CTL escape[12,14,15], although this implies that CTL-mediated killing of virus-infected cells is not of great importance during this phase of infection.

Although many examples of mutations in epitopes recognized by CTLs have been described[8-12], there have been limited longitudinal data to show variant viruses escaping to fixation[9] and this may be the cause of some of the controversy in this area[9,12,14,15]. We describe two clear examples of virus escape caused by the same mutation in an immunodominant epitope that alters binding to the presenting HLA molecule, HLA-B27. In both patients, escape to fixation was observed as they progressed to AIDS. This shows that CTL responses can still exert strong selective pressure late in infection and suggests that immune escape may contribute to the late collapse of the immune control in HIV infection.

CTL response in donor 007

Donor 007 was studied in most detail[7-9,16,17]. He is a hemophiliac who was infected by contaminated factor VIII in 1983. His CTL response has been dominated by the HLA-B27-restricted epitope in p24 Gag (HIV_{LAI} Gag amino acid residues 263–272), sequence KRWIIMGLNK[16], through the 9 years of study. Only Gag-expressing targets were recognized by his CTL activated by autologous virus (Fig. 1*a*). Lysis of Gag-vaccinia-infected chromium-51-labeled targets was completely inhibited by unlabeled ("cold") targets when the cold targets were pulsed with the peptide KRWIIMGLNK, indicating that the CTL response in donor 007 was monospecific (Fig. 1*b*). A "fresh" response [killing by unstimulated peripheral blood mononuclear cells (PBMCs)] to this peptide has been consistently observed in this donor. Such responses indicate a high level of circulating

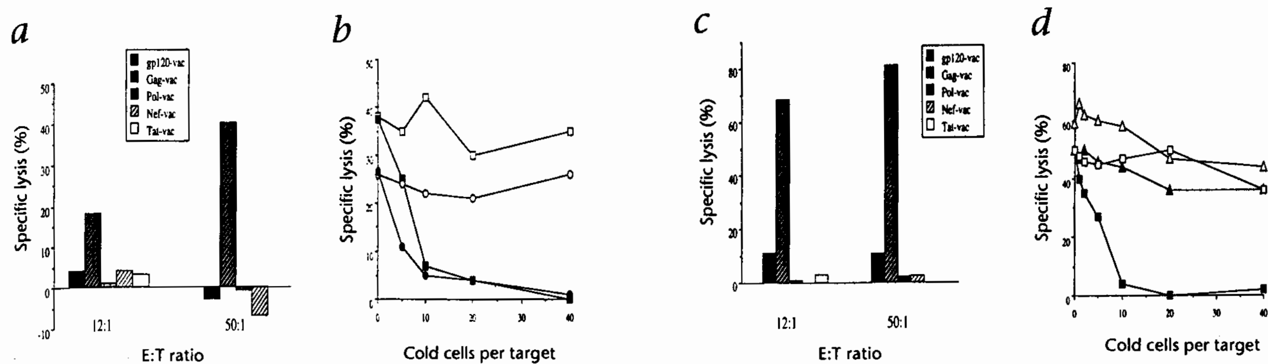

Fig. 1 Immunodominant CTL responses in donors 007 and 049 to the HLA-B27-restricted Gag epitope 263–272. *a*, CTL from of donor 007 only recognize targets expressing Gag. Bulk culture lymphocytes were assayed against recombinant vaccinia-infected autologous B lymphoblastoid cell (BCL) targets. Percent specific lysis calculated by substraction of lysis of control vaccinia-infected targets. *b*, CTLs from donor 007 only recognize the peptide epitope KRWIIMGLNK within Gag. Cold target inhibition assay. "Hot" targets: squares show HLA-B2705-matched BCLs, circles show autologous BCLs, infected with Gag-vaccinia and labeled with ^{51}Cr, as in *a*. Cold targets: HLA-B27-matched targets pulsed without (○) and with (●) peptide KRWIIMGLNK; autologous BCLs pulsed without (□) and with peptide (■). Effectors: bulk cultured lymphocytes from donor 007, effector:target ratio 20:1. Lysis of control vaccinia-infected targets (subtracted): 10%. *c*, CTLs from donor 049 only recognize targets expressing Gag. Bulk culture lymphocytes were assayed against autologous BCLs as in *a*. Lysis of control vaccinia-infected targets has been subtracted. *d*, CTLs from donor 049 only recognize the peptide epitope KRWIILGLNK within Gag. Hot targets: Gag-vaccinia-infected, ^{51}Cr-labeled autologous BCL (HLA class I type: A3/- B27/39 Cw1/12). Cold targets: autologous BCLs pulsed without (□), with peptide KRWIILGLNK (■, △), and with influenza-specific HLA-B27-restricted peptide SRYWAIRTR (▲); Effectors: 049 bulk cultured lymphocytes, E:T 50:1, in all cases except △: HLA-A3-restricted p17 Gag peptide RLRPGGKKK-specific CTL line from donor 003 (HLA class I type: A2/3 B7/51 Cw7/14). Lysis of control vaccinia targets subtracted (11% for 049 bulk CTLs, 0% for 003 CTL line).

effector CTLs, which have been shown to be oligoclonal[7].

After 12 years of asymptomatic infection, with a slowly declining CD4 count and stable CTL response, the CD4 count in donor 007 declined rapidly to 60 cells/µl (Fig. 2*a*). The CTL response seen in fresh PBMCs and bulk cultured lymphocytes from donor 007 was still present at this time, although at a weaker level than seen previously (Fig. 2*b*). Fresh killing was detectable only after incubation with targets for 12 hours or more, rather than the conventional 4 hours. At this time, his RNA virus plasma load was very high (530,965 copies/ml). Frozen samples from 38 months before this and earlier had shown levels of virus at or below the threshold of detection (<100–500 copies/ml) (Fig. 2*a*).

Variation within the immunodominant epitope in donor 007

Autologous provirus sequences from activated T cells from donor 007 that encode this immunodominant epitope were followed over time (Fig. 2*c*, Table 1). Although the CD4 count, viral load and CTL response were relatively stable, little sequence variation was observed; his virus showed the relatively unusual methionine residue, rather than leucine, at position 268. However, when the CD4 count fell sharply, a proportion of the autologous provirus showed a lysine-for-arginine change at the anchor position 2 in the epitope. This proportion increased to 100% so that all of the proviral DNA sequenced now encodes the epitope sequence KKWIIMGLNK (K2M6).

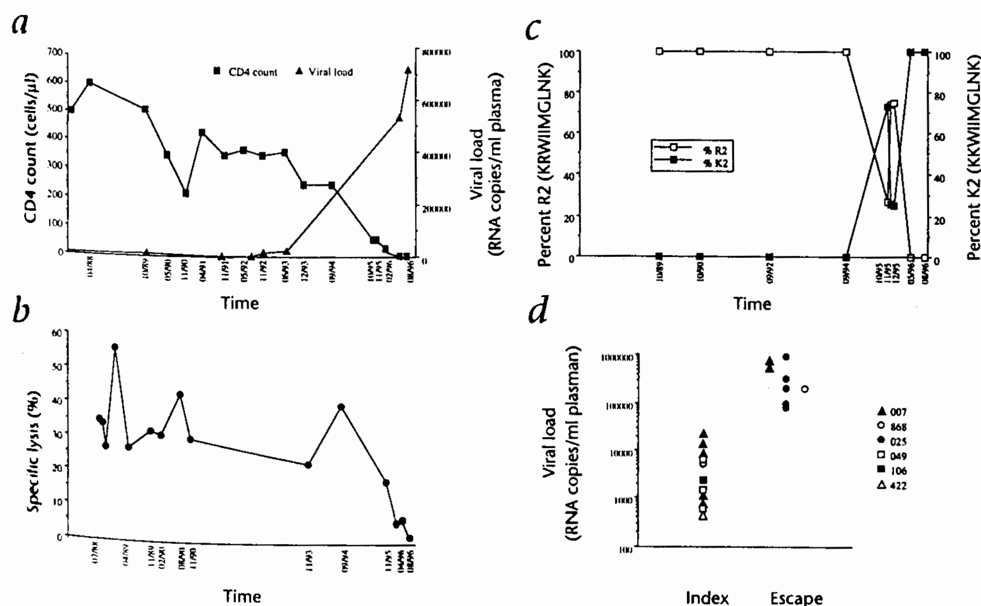

Fig. 2 *a*, Serial CD4 counts and HIV-1 viral loads of donor 007. *b*, Loss of fresh CTL response 12–13 years following infection in donor 007. Serial "fresh" responses in unstimulated PBMCs. E:T ratios 25:1 to 40:1. Supernatants assayed at 4–6 h. *c*, Appearance of K2 variant 12 years following infection in donor 007. Serial frequencies of index peptide sequence KRWIIMGLNK (R2) and variant sequence KKWIIMGLNK (K2) encoded by proviral DNA from donor 007. *d*, Appearance of CTL escape mutants in the HLA-B27 Gag epitope is associated with high virus load (P = 0.025).

Table 1 Variation in HLA B27-restricted HIV-1 gag epitope KRWIILGLNK in six responders

Donor (Year infected)	Date	Percent variant					CD4 (cells/μl)	Load (RNA copies/ ml plasma)
		R2L6	R2M6	R2I6	K2L6	K2M6		
007 (1983)								
	07/84	–	–	–	–	–	–	1,811
	10/89	0	100	0	0	0	510	8,650
	10/91	10	90	0	0	0	350	1,019
	07/92	0	100	0	0	0	400	778
	11/92	–	–	–	–	–	350	14,331
	06/93	–	–	–	–	–	360	21,368
	09/94	0	100	0	0	0	250	–
	10/95	0	27	0	0	73	60	–
	11/95	0	74	0	0	26	60	–
	12/95	0	75	0	0	25	60	–
	05/96	0	0	0	0	100	10	530,965
	08/96	0	0	0	0	100	10	716,853
049** (1983)								
	11/84	–	–	–	–	–	–	1,458
	11/90	–	–	–	–	–	410	144
	05/91	100	0	0	0	0	710	–
	07/92	–	–	–	–	–	680	1,387
	01/93	–	–	–	–	–	660	588
	04/95	100	0	0	0	0	820	–
	04/96	100	0	0	0	0	580	6,650
	08/96	–	–	–	–	–	370	5,388
422 (1987)								
	01/90	100	0	0	0	0	920	–
	01/93	100	0	0	0	0	1380	–
	10/94	100	0	0	0	0	1190	–
	03/96	100	0	0	0	0	1040	–
	08/96	–	–	–	–	–	940	417
106 (1989)								
	04/95	0	96	4	0	0	200	–
	04/96	0	100	0	0	0	130	2,201
	08/96	–	–	–	–	–	110	21,790
868 (1989)								
	11/95	0	100	0	0	0	330	–
	04/96	0	56	44	0	0	230	193,568
	06/96	0	100	0	0	0	430	5,142*
	08/96	–	–	–	–	–	430	5,400*
025** (1983)								
	11/84	–	–	–	–	–	–	2,622
	05/91	95	5	0	0	0	200	–
	01/93	0	0	0	0	100	90	83,235
	07/93	0	0	0	0	100	60	91,895
	01/96	0	0	0	5	95	0	881,948
	05/96	0	0	0	0	100	0	301,788
	08/96	0	0	0	0	100	0	200,848

*Started AZT/ddI 05/96.
**049 and 025 are hemophiliac brothers, and were exposed to the same batches of contaminated factor VIII.

Recognition of HLA-B27-matched cells pulsed with the K2M6 variant by fresh CTLs from donor 007 was reduced compared with the index (R2M6) sequence, but apparently not entirely abrogated (Fig. 3*b*). However, when target cells were washed and left for various times after the addition of peptide, we observed that the K2 peptide–HLA-B27 complexes were very unstable compared with the R2 index peptide–HLA-B27 complexes. The R2 index, like other epitope peptides (23–25 and P.J.R.G. *et al.*, manuscript submitted), formed targets that were recognized by CTLs more than 20 hours later, whereas the K2 variant failed to bind adequately for more than 2 hours (Fig. 3*c*). When the K2 peptide was used to pulse target cells immediately before an assay with CTL clones, there was good recognition of the epitope (Fig. 3*d*), suggesting that the overall orientation of the peptide in the binding groove was not altered by the R2 to K2 change.

Intracellular presentation of KKWIIMGLNK by HLA-B*2705

The critical question was whether the affinity of the variant K2M6 epitope for HLA-B27 was sufficient for this peptide to be presented via the usual cytosolic-to-endoplasmic route, or whether this mutation could prevent presentation of this immunodominant epitope. To address this, minigenes encoding the R2M6 index sequence or the K2M6 peptide plus a control HLA-B27-restricted influenza nucleoprotein 384–392 epitope (SRYWAIRTR) were constructed and transfected into target cells. Cells transfected with the R2M6 minigene were recognized by both the Gag-specific CTLs and the influenza-specific CTLs. In contrast, cells transfected with the K2M6 minigene were seen by the influenza-specific CTLs alone (Fig. 3*d*). Recognition of the influenza epitope showed that the gene was expressed, but that there was inadequate presentation of the K2M6 variant epitope. Therefore the K2M6 sequence represents a CTL escape variant.

Binding of KRWIIMGLNK and KKWIIMGLNK to HLA-B*2705

Arginine at position 2 in the epitope is one of two residues that bind or "anchor" peptides to the HLA-B27 molecule and is an essential component of the HLA-B27 peptide binding motif[18] locating in the B pocket. All 24 peptides eluted from C1R-HLA-B27 cells[19,20], and all of seven HLA-B2705-restricted CTL virus epitopes described[21,22], had arginine in this position. Was the mutated epitope (K2M6) even capable of binding to HLA-B27? When the altered peptide was used to stabilize HLA-B27 in cell lysates, it was found that some binding occurred, but only at high concentrations of peptide, in contrast to the index peptide (Fig. 3*a*). From these binding assays, the estimated relative K_d for the index R2 peptide is at least 50 to 100 times that of the K2 variant.

CTL responses and epitope variation in HLA-B27-positive donors

To investigate further the relation between HLA-B27-restricted CTL responses and epitope variation, we studied five other HIV-seropositive donors with HLA-B*2705. In all five, a strong CTL response to the same HLA-B27-restricted epitope within p24 Gag was identified, and in three of these five donors, this was the immunodominant or single response. Bulk cultured lymphocytes from donor 049 recognized only targets expressing Gag (Fig. 1*c*) and no peptides within Gag other than KRWIILGLNK (Fig. 1*d*). Similarly narrow CTL responses were identified

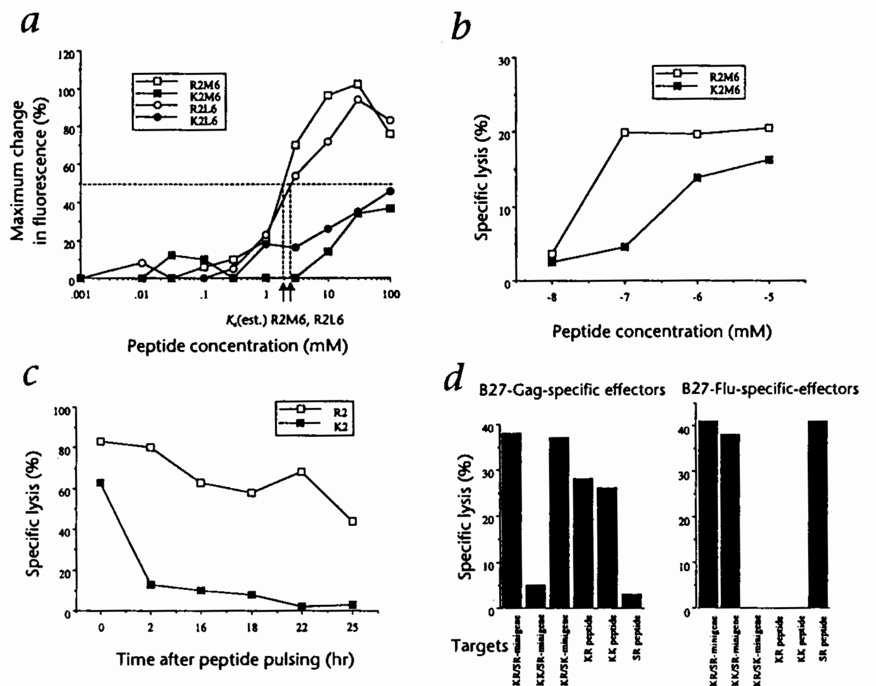

Fig. 3 *a*, K2 variant peptide binds only very inefficiently compared with the binding of R2 index peptide. Binding of KXWIIXGLNK peptides to T2.B2705. □, R2M6; ■, K2M6; ○, R2L6; ●, K2L6. Estimated relative K_d [K_d(est.)] for each peptide derived from concentration at which half-maximum (50%) change in fluorescence is achieved. *b*, Variant K2 peptide is not well-recognized by fresh PBMCs from donor 007. Fresh killing of peptide-pulsed autologous BCL targets by PBMCs from donor 007 (after appearance of K2 mutant virus): supernatant assayed at 14 h. Lysis of targets pulsed with no peptide 4% subtracted. *c*, Peptide-B2705 complexes are very unstable when exogenously added peptide is K2 variant compared with R2 index. Lysis of autologous BCL targets by CTLs from donor 049. Targets were pulsed with 10 µM peptide, then washed twice and incubated for the periods shown before addition of CTLs. The peptide was either R2 index (KRWIILGLNK) or K2 variant (KKWIILGLNK). *d*, HLA-B2705 molecules are not adequately loaded with the variant K2 peptide in the endoplasmic reticulum to sensitize target cells for lysis by CTLs. Lysis of targets transfected with pCEP4 expressing no peptide subtracted to calculate percent specific lysis (13% for Gag-specific effectors, 8% for "influenza-specific effectors"). KR/SR minigene: targets transfected with minigene expressing the peptide: metKRWIIMGLNKIVELRSRYWAIRTR; KK/SR minigene: targets transfected with minigene encoding metK<u>K</u>WIIMGLNKIVELRSRYWAIRTR; KR/SK minigene: encoding metKRWIIMGLNKIVELRS<u>K</u>YWAIRTR. Peptides (10 mM) used in assay: R2 - KRWIIMGLNK; K2 - K<u>K</u>WIIMGLNK; SR - SRYWAIRTR.

in donor 422 (data not shown). In two donors whose CD4 count was declining, a broader CTL response was observed: eight and six epitopes were recognized by bulk cultured lymphocytes from donor 868 (whose dominant and only fresh CTL response was also to the KRWIIMGLNK peptide), and donor 106, respectively (data not shown). In donor 025, whose CD4 count had fallen to zero, CTL responses were no longer detectable, but bulk CTL responses to KRWIILGLNK had been documented on previous occasions[26]. The immunodominance of this response in donor 025 was not established. Proviral DNA encoding the HLA-B27-restricted epitope was sequenced in these five donors (Table 1), and little variation was observed in HLA-B27-positive donors 049, 422 and 106, whose viral load was low (Table 1). Similarly, no stable epitope variation affecting CTL recognition was observed in four HLA-B27-positive donors whose viral loads were not documented but whose CD4 counts were high (414–900/µl)[12].

In donor 868, whose recent viral load was high (193,568 copies/ml), some variation at position 6 within the epitope (isoleucine for methionine) was seen. This variant (I6) was not recognized by fresh CTLs from this donor and also antagonized the CTL response to the index peptide (not shown). Following commencement of antiviral therapy, the viral load fell to 5000 copies/ml and coincidentally the escape variant disappeared. The M6 variant observed in several donors was well recognized by CTL from donors 007, 868 and 106 in whom this variant was found (data not shown).

In donor 025, the identical arginine-to-lysine escape mutation occurred at position 2 in the epitope as was seen in donor 007. The appearance in donor 025 of this K2M6 variant coincided with a decline in the CD4 count from 200 cells/µl to 90 cells/µl, and a rapidly rising viral load (83,235 to 881,948 RNA copies/ml over 36 months, Table 1). Noncoding nucleotide substitutions in sequences from 007 and 025 show that the viruses in the two individuals are distinct.

This arginine-to-lysine change was not observed in provirus sequences from HLA-B2705-negative donors who have progressed to AIDS (ref. 1, 27). We observed no sequence variation within this epitope in more than 200 clones of proviral DNA from seven out of seven HLA-B2705-negative donors (virus load range: 7,306–346,008 RNA copies/ml; CD4 count range 0 to 510/µl: not shown). Furthermore, this lysine-for-arginine change is not observed at position 2 in the epitope in any of the 32 B clade sequences listed in the Los Alamos database: arginine is present in this position in all sequences listed[27].

Discussion

In both donors 007 and 025, the arginine-to-lysine mutations occurred late in the course of infection and were ultimately found in 100% of the provirus. These were not recognized by the dominant CTLs and must therefore be escape mutations, but why did it take 9–12 years for these to acquire a selective advantage, when more than 10^9 virions are generated each day[28,29]? One possibility is that the K2 mutant may only have a selective advantage when unequivocal immunodeficiency is present. When the CD4 count is high and the viral load is low, there could be several forces acting against the virus. These include lytic and nonlytic T-cell responses[30,31], as well as other immune responses. It is possible that CTL-mediated nonlytic immune control by chemokines is the dominant selective force controlling HIV during the asymptomatic phase of infection, CTL-mediated lysis becoming dominant toward the end of this phase. The switch of viral phenotype from non-syncytium-inducing (NSI) to syncytium-inducing (SI), which frequently occurs at this time, may contribute to CTL-mediated cytotoxicity assuming the dominant role, as the chemokines RANTES (restricted upon activa-

tion, normal T cell expressed and secreted), and MIP-1α and MIP-1β (macrophage inflammatory proteins) only block the entry of NSI viruses into CD4$^+$ cells[31]. CTL escape mutants would not be preferentially selected in viruses already susceptible to chemokines.

Alternatively, during the long asymptomatic period, the monospecific CTL response observed in donors 007 and 049 may have been sufficiently potent to clear target cells expressing the R2 index and the K2 variant equally efficiently, even when the K2 variant is expressed for brief periods of time on the surface of infected cells, but this capacity may be undermined by the general decline of immune competence. This explanation is supported by the observation that clones that recognize and lyse targets rapidly, within the 4-hour time course of a standard assay, may not distinguish between the K2 variant and the R2 wild type (Fig. 3*d*). On the other hand, effectors that recognize and lyse peptide-pulsed targets more slowly, only after more than 12 hours incubation in the assay (Fig. 2*b* and 3*b*), preferentially recognize the R2 index peptide, presumably because by this time the K2 variant is no longer bound (Fig. 3*c*). Against this is the failure of cells transfected with the K2 minigene to load HLA-B27 molecules with the K2 epitope adequately (Fig. 3*d*).

A third possible explanation for the late appearance of CTL escape mutants is that a series of compensatory mutations may have been required elsewhere in the genome in addition to the R2 to K2 change in order to maintain the stable structure of p24 Gag. The rarity of lysine in this position in B clade viruses (0 out of 32 sequences), but the presence in 3 out of 34 A clade sequences and 1 out of 10 A clade sequences[27] gives support to this idea. The p24 Gag structure recently reported[32] shows the epitope positioned in a central, conserved region of the molecule.

This study shows that in HLA-B27 asymptomatic seropositive donors, the CTL response is normally dominated by recognition of the Gag 263–272 epitope, sometimes to the exclusion of other CTL responses. This contrasts with those CTL responses restricted by other HLA types, such as HLA-B8. In the latter, CTL responses are broader and more variable, and epitope variation occurs even in asymptomatic individuals who may have a low viral load[8,9]. The identification of HLA-B27 as the HLA class I molecule most closely associated with nonprogression in HIV infection[33,34], and HLA-B8 as associated with rapid progression[33,34], may be related to these differences in immune response.

Our results show that viruses capable of evading a previously stable immunodominant CTL response became detectable for the first time as the immune system finally decays. This evidence implies that the escalating viremia associated with the onset of AIDS may be driven by viruses capable of immune evasion. Escape from CTL responses may play a more important role in the progression of HIV infection than has been recognized.

Methods

Patients. Donor 007 is a 38-year-old hemophiliac infected as a result of receiving contaminated factor VIII in 1983. He has been asymptomatic to date. Donors 007, 025 and 049 are hemophiliacs also infected in 1983–1984. Donors 422, 868 and 106 have homosexually acquired infection. Donor 025 alone has been symptomatic of HIV infection; he was treated twice for episodes of *Pneumocystis carinii* pneumonia and died in November 1996. Donor 868 has had generalized lymphadenopathy since 1993. Donor 868 is the only one to receive antiretroviral therapy [zidovudine (AZT) + dideoxyinosine (ddI)], which was initiated midway through this study (May 1996).

Generation of bulk cultured CTLs and CTL assays. Bulk cultured lymphocytes and CTL assays were set up as previously described[1]. Briefly, PBMCs were cocultured with autologous phytohemagglutinin (PHA)-stimulated lymphoblasts. Lymphocult T (Biotest) was added to the medium from day 7 onward. Assays were performed on bulk cultured lymphocytes from day 14 to 25. Vaccinia infections were done at 3 plaque-forming units (PFU) per cell, followed by 16 h incubation and target labeling with ^{51}Cr. A vaccinia-influenza PB2 construct was used to infect control targets. Targets were incubated at 5000 targets per well in 100 µl, and either effectors, medium or 5% Triton X-100 was added in duplicate wells to a total volume of 200 µl. Supernatant was assayed at 4–6 h (except in Fig. 3*b*) and specific lysis determined as 100 × (experimental lysis – spontaneous lysis)/(maximum lysis – spontaneous lysis).

Viral load measurement. Plasma obtained from EDTA blood samples was frozen at –70 °C within 6 h of the blood sample being taken. Viral load was measured using the Roche Amplicor RT-PCR kit (Roche Diagnostic Systems, Branchburg, NJ). Samples taken before 1994 for which viral load measurements were made were stored at –20 °C or –40 °C.

Proviral DNA sequencing. DNA was extracted using a Puregene DNA Isolation Kit (Gentra Systems, Research Triangle Park, NC) from PHA-stimulated lymphocytes, which had been cultured for 2–3 days to enrich for competent provirus. Primers for the PCR were 5'–ATCAATGAGGAAGCT–3' and 5'–TCCTTTCCACATTTCCAAC–3' (primary reaction); 5'–GATAGATCTCAAATAGGATGGATG–3' and 5'–GCTAGAATTCCTGACATGCTGTCA–3' (secondary reaction). Genomic DNA (1 µg) was added to buffer containing 2.5 U *Taq* polymerase, 0.05 M KCl, 0.01 M Tris pH 8.0, 4.5 mM $MgCl_2$ and 0.2 mM each of dNTPs. Cycling conditions were 5 min at 94 °C followed by 30 cycles at 94 °C (1 min), 50 °C (1 min) and 72 °C (30 s) for the primary reaction. The primary product (1 µl) was used in the secondary reaction, for which the conditions were identical, except that the concentration of $MgCl_2$ was 3 mM and the annealing temperature was 53 °C. The product was purified, ligated into a T-vector using the pMOS Blue T-vector Kit (Amersham Life Science, Little Chalfont, Buckinghamshire, UK) and sequenced[1]. Between 10 and 24 clones from each time point were sequenced.

Binding assays. Peptide binding to HLA-B*2705 was quantified by using a T2 FACS stabilization assay (R.A.C., unpublished data). Transporter-deficient T2 cells transfected with B2705 (T2.B2705)[21] were cultured at a density of 1 × 10^6 per ml in RPMI 1640 containing 5% calf serum and 0.5 mg/ml G418. Cells were washed twice with RPMI 1640 and resuspended in EX-CELL 301 (IRH Biosciences, Lenexa, KS) defined serum-free media supplemented with 2 mM L-glutamine, nonessential amino acids, and sodium pyruvate. T2.B2705 cells (5 × 10^5) were added in a volume of 100 µl to an equal volume of peptide (or control diluent) in a 96-well U-bottom plate (Costar, Cambridge, MA). Peptides were diluted in RPMI 1640 from a stock prepared at 10 mg/ml in DMSO. Incubations were for 16–18 h at 37 °C in a 5% CO_2 incubator. After incubation, cells were transferred to microfuge tubes and stained for flow cytometric analysis. Cell-surface B2705 molecules were detected with a combination of saturating amounts of ME.1 (specific for HLA-B27, -B7, -Bw22) and affinity-purified fluorescein isothiocyanate (FITC)-conjugated F(ab')$_2$ goat anti-mouse IgG (Fc-specific; Organon Technika Corp., West Chester, PA) antibodies. Samples were analyzed using a FACScan (fluorescence-activated cell sorter, Becton-Dickinson, Palo Alto, CA) flow cytometer, and the mean fluorescence intensity (MFI) of a minimum of 5000 events was calculated using Lysis II software (Becton Dickinson). Maximum peptide binding was assessed with 100 µM of the influenza A nucleoprotein peptide SRYWAIRTR[23]. The relative binding of variant peptides was compared with the percent change in fluorescence determined as follows: percent maximum change in fluorescence = 100 × (MFI for test peptide – MFI for diluent control)/(MFI for SRYWAIRTR – MFI for diluent control).

Minigene construction and cell transfection. Minigenes encoding the 26 amino acid peptides metKRWIIMGLNKIVELRSRYWAIRTR, metK<u>K</u>WIIMGLNKIVELRSRYWAIRTR and metKRWIIMGLNKIVELRS<u>K</u>YWAIRTR were constructed by cloning two sets of annealed oligonucleotide pairs encoding metK<u>X</u>WIIMGLNKIV and LELRS<u>X</u>YWAIRTR into the *Hin*dIII–*Bam*HI site of pCEP4 (Invitrogen, San Diego, CA). Each oligonucleotide pair contained single-stranded 5' overhangs compatible with either *Hin*dIII or *Bam*HI on

one end, and *XhoI* on the other. The three fragments were ligated, and clones sequenced to confirm the expected minigene sequence, orientation, and absence of concatemerization. C1R cells stably transfected with HLA-B2705 and pSV2neo (G418 resistance) were transfected with the minigenes or pCEP4 alone, and positive transfectants selected in RPMI supplemented with 10% fetal calf serum and hygromycin (1.5 mg/ml), then maintained in the same medium supplemented with G418 (0.5 mg/ml)[23].

Statistics. In the comparison of sequences containing CTL escape variants and index sequences in the association with viral load, the *P* value was obtained by a standard *t*-test that compared mean viral loads for each donor before epitope mutation, with mean viral loads in donors 007 and 025 after mutation. Data from donor 868 was excluded (on antiretroviral therapy midway through study).

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