Immune Escape Precedes Breakthrough Human Immunodeficiency Virus Type 1 Viremia and Broadening of the Cytotoxic T-Lymphocyte Response in an HLA-B27-Positive Long-Term-Nonprogressing Child

M. E. Feeney,[1,2]* Y. Tang,[1] K. A. Roosevelt,[1] A. J. Leslie,[3] K. McIntosh,[2] N. Karthas,[2] B. D. Walker,[1,4] and P. J. R. Goulder[1,3]

Partners AIDS Research Center and Infectious Disease Division, Massachusetts General Hospital and Harvard Medical School,[1] and Children's Hospital,[2] Boston, Massachusetts; Department of Paediatrics, Nuffield Department of Medicine, University of Oxford, Oxford, United Kingdom[3]; and Howard Hughes Medical Institute, Harvard Medical School, Boston, Massachusetts[4]

The emergence of cytotoxic T-lymphocyte (CTL) escape mutations in human immunodeficiency virus type 1 (HIV-1) proteins has been anecdotally associated with progression to AIDS, but it has been difficult to determine whether viral mutation is the cause or the result of increased viral replication. Here we describe a perinatally HIV-infected child who maintained a plasma viral load of <400 copies/ml for almost a decade until a nonbinding escape mutation emerged within the immunodominant CTL epitope. The child subsequently experienced a reemergence of HIV-1 viremia accompanied by a marked increase in the number of CTL epitopes targeted. This temporal pattern suggests that CD8 escape can play a causal role in the loss of immune control.

Selection for viral sequence mutations which allow human immunodeficiency virus type 1 (HIV-1) to evade recognition by cytotoxic T lymphocytes (CTL) has been well documented in humans and in animal models (2, 3, 6, 7, 9). However, it is likely that CTL responses vary in their antiviral efficacies, and the timing of escape and its consequences for immune control are also likely to differ among epitopes (10). A compelling demonstration of the relationship between HIV-1 mutational escape and loss of immune control of viremia has come from studies of the highly immunodominant Gag CD8 epitope KRWIILGLNK (KK10; Gag amino acids 131 to 140) (6, 7, 9) presented by HLA-B27, which is consistently associated with slowly progressive HIV disease (8, 13). The emergence of mutations within this epitope has been shown to coincide with increased viral replication and progression to AIDS (7, 9). However, it has been difficult to discriminate cause from effect; viral mutation within this epitope may be the critical event which allows the virus to escape immune control, leading to breakthrough viremia, or the mutation may emerge as a stochastic function of increased viral replication due to some other cause. Here we describe a perinatally HIV-infected child expressing HLA-B27 who maintained a plasma viral load below the limit of detection for many years on minimal antiviral therapy until breakthrough viremia occurred at 9 years of age. Sequencing of autologous virus from samples obtained shortly before this increase in viremia, while the viral load was still <50 copies of RNA/ml of plasma, demonstrates the emergence of a minor population of viral clones possessing a characteristic B27-KK10 escape mutation.

Patient TCH-043 was diagnosed with asymptomatic HIV-1 infection at 27 months of age. A single HIV RNA measurement performed on plasma obtained prior to antiviral therapy revealed a viral load of 307 copies/ml. The subject subsequently began dual-nucleoside therapy with zidovudine and didanosine, and all HIV-1 RNA measurements over the next 7 years were either below the limit of detection or measurable at fewer than 400 copies/ml (Fig. 1, top panel). A detailed study of the subject's HIV-specific CTL response was performed when the patient was 7.4 years old, during the period of fully suppressed viremia (previously described in reference 6). Recognition of defined HIV-1 optimal epitopes and overlapping peptides spanning Gag, reverse transcriptase (RT), Nef, and gp41 (15-mers with 10- to 11-amino-acid overlap, based on the SF2 sequence) was assessed in a gamma interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assay with 10 μM peptide and 50,000 to 200,000 peripheral blood mononuclear cells (PBMCs) per well, as previously described (5). The subject displayed a highly immunodominant IFN-γ response to the B27-restricted Gag epitope KRWIILGLNK (B27-KK10; 1,925 SFC/10^6 PBMCs). A second response of considerably lower magnitude, also restricted by HLA-B27, targeted Gag epitope IRLRPGGKK (B27-IK9; 192 SFC/10^6 PBMCs). No other responses were detected in Gag, RT, Nef, or gp41. Retrospective testing of cryopreserved PBMCs obtained at the time of diagnosis revealed that the response to B27-KK10 was already present when the patient was 27 months of age; B27-IK9 and A26-EL9 were not recognized at this early time point (Fig. 1, middle panel).

When the patient was 9 years old, marked changes in the HIV-specific CTL response occurred. The frequency of IFN-γ-secreting T cells specific for B27-KK10 declined precipitously (Fig. 1, middle panel). During this interval, immunodominance shifted to two other epitopes within Gag: the

* Corresponding author. Mailing address: Partners AIDS Research Center, 5th Floor, 149 13th St., Charlestown, MA 02129. Phone: (617) 726-6126. Fax: (617) 726-5411. E-mail: mfeeney@partners.org.
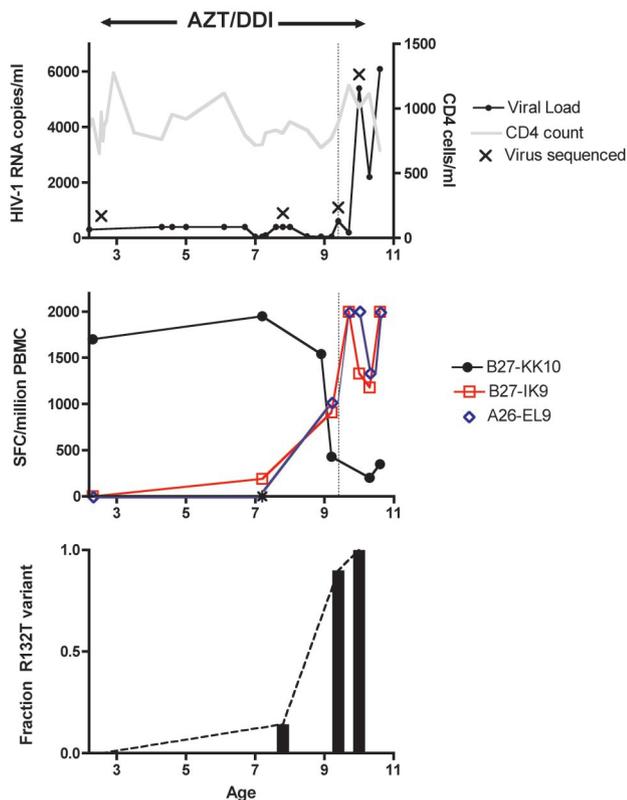
FIG. 1. Longitudinal clinical, CTL, and viral sequence data for patient TCH-043. AZT, zidovudine; DDI, didanosine; SFC, spot-forming cells. (Top panel) Longitudinal changes in viral load and CD4 count from time of diagnosis to present. Where viral load measurements were beneath the limit of detection, the threshold value is shown. Viral sequencing was performed at the time points indicated (×), given in years of patient age. (Middle panel) Longitudinal changes in CTL recognition of three immunodominant epitopes were assessed by an IFN-γ ELISPOT assay. A26-EL9 was not tested when the patient was 7.2 years old, but a 17-mer containing this epitope (AFSPEVIPMFSALSEGA; indicated by an asterisk) was not recognized. The dotted vertical line indicates the earliest measurable return of viremia (640 copies/ml) at age 9.4 years. (Bottom panel) Fraction of autologous viral sequences containing the R132T mutation.

previously subdominant B27-restricted epitope IRLRPGGKK (B27-IK9) and the previously unrecognized A26-restricted epitope EVIPMFSAL (A26-EL9). Comprehensive screening using overlapping peptides spanning all HIV-1 proteins revealed that the CTL response had broadened considerably to target at least 17 distinct specificities (Fig. 2). Subsequently, after 7 years of stable viral control, an increase in viral load to 640 copies/ml of plasma was detected. Viremia persisted over the next 7 months, and the viral load increased to 5,400 copies/ml. Antiviral therapy was discontinued, with no significant effect on the subsequent viral load measurements (<0.5 log change).

To determine whether viral sequence changes mediated by immune or drug selection pressure coincided with the reemergence of viremia, longitudinal HIV-1 sequencing was performed on samples obtained at initial presentation (age, 2.2 years), shortly before viral breakthrough (age, 7.8 years), and following the increase in viral replication (ages, 9.4 and 10 years). At the age of 7.8 years (viral load, <50 copies/ml), the HIV-1 sequence could be determined only following culture of virus from CD8-depleted PBMCs. HIV-1 RNA was extracted directly from plasma at all other time points. Nested PCR was performed using primers and conditions previously described (5), followed by TA cloning (TOPO TA cloning kit; Invitrogen, Carlsbad, Calif.), DNA purification (QiaPrep Turbo Miniprep system; QIAGEN, Valencia, Calif.), and sequencing on an ABI 3700 DNA analyzer from Applied Biosystems.

Following breakthrough viremia, all 40 viral clones possessed an arginine-to-threonine substitution at position 2 within B27-KK10 (R132T) (Fig. 3, top panel). This R132T mutation has previously been shown to prevent binding of KK10 to the HLA-B27 molecule (6) due to loss of the critical P2 anchor residue. Interestingly, this R132T mutation predated the increase in viral replication, as it was already present in 3 of 14 clones isolated from the patient at 7.8 years of age, while plasma viremia remained undetectable (Fig. 3, top panel). Furthermore, phylogenetic analysis indicates that the viral escape
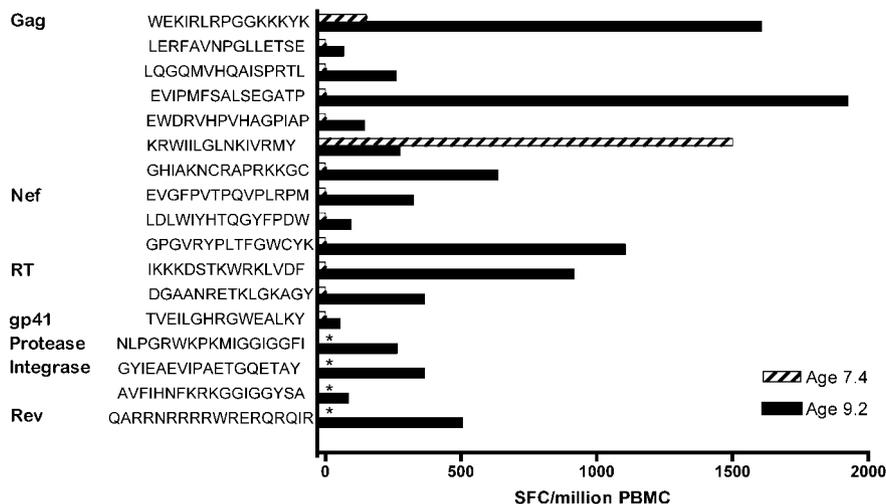
FIG. 2. Broadening of CTL response precedes viral breakthrough. Recognition of overlapping 15-mer peptides spanning clade B HIV-1 Gag, Nef, RT, and gp41 was assessed by an IFN-γ ELISPOT assay when the patient was 7.4 years old (2 years before viral breakthrough) and 9.2 years old (2 months before viral breakthrough). In addition, 18-mer peptides spanning all other HIV-1 gene products (gp120, Rev, Tat, Vpr, Vpu, Vif, protease, and integrase) were tested when the patient was 9.2 years old but not at 7.4 years of age. Missing data are indicated by asterisks.

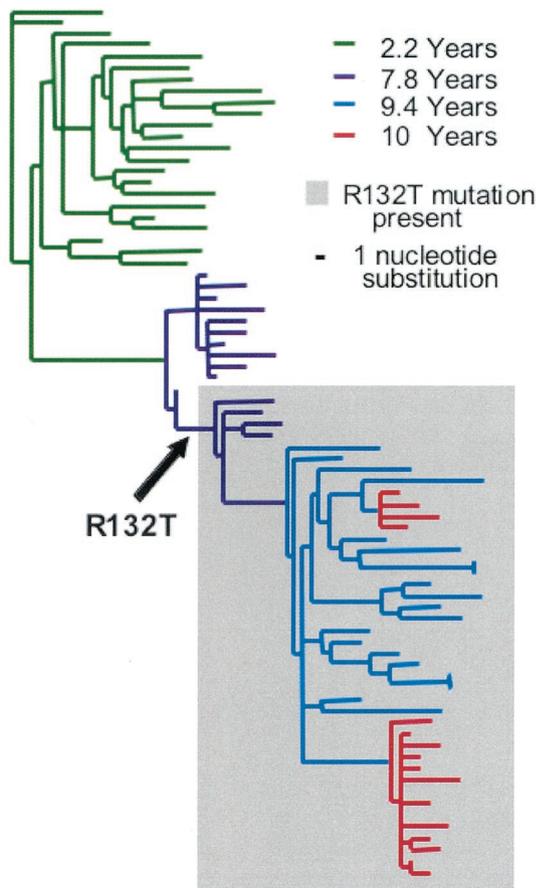| Subject	Gag B27-KK10										Clones
Mother of TCH-043	K	R	W	I	I	L	G	L	N	K	
TCH-043 Age 2.2	-	-	-	-	-	-	-	-	-	-	14/19
						M					2/19
					I						2/19
					T	M					1/19
Age 7.8	-	-	-	-	-	M	-	-	-	-	10/14
		T									3/14
	-	-	-	-	-	-	-	-	-	-	1/14
Age 9.4	-	T	-	-	-	-	-	-	-	-	12/19
		T				M					6/19
		T	R			M					1/19
Age 10	-	T	-	-	-	-	-	-	-	-	21/21

FIG. 3. Longitudinal HIV-1 sequence changes in B27-KK10. (Top panel) Longitudinal changes within the Gag epitope B27-KK10 were determined by clonal sequencing of samples from patient TCH-043 and the subject's mother. The R132T mutation in B27-KK10 was present in a minority of clones when the patient was 7.8 years old but became the predominant species at the time of viral breakthrough at age 9.4 years. Minor variants are shown in gray. The number of clones with given mutations per total number of clones is given in the final column. (Bottom panel) Maximum likelihood phylogenetic tree showing the evolution of HIV in patient TCH-043 over time. Branch color corresponds to the time point at which each clone was isolated (given as age of patient TCH-043 in years). All viral clones within the shaded box possess the R132T mutation. All branch lengths are drawn to scale.

sequences that predominated after viral breakthrough are likely to have originated from clones displaying R132T (Fig. 3, bottom panel), suggesting that this mutation conferred a strong selection advantage. Although several RT mutations conferring resistance to nucleoside analogues were detected

following viral breakthrough (V118I, 215Y, 211K, 210W, and M41L), each of these mutations was already present when the patient was 7.8 years old, while viremia remained undetectable (11).

Mutational escape from the KRWIILGLNK epitope has previously been shown to coincide with the emergence of viremia among B27-positive subjects after many years of spontaneous viral control. However, in these cases, limited sample availability prevented determination of the precise timing of CTL escape relative to the loss of viral control. Therefore, it has been suggested that the emergence of the viral variant may have been the result of increased viral replication rather than a causal contributor to breakthrough viremia. The longitudinal data presented here demonstrate that escape from the immunodominant CTL response clearly preceded breakthrough HIV-1 viremia in this long-term-nonprogressing child. The decline in the magnitude of the KK10 response and the shift of immunodominance to other epitopes occurred prior to any measurable return of viremia. Although the subject had been receiving long-term nucleoside analogue therapy, no RT mutations emerged coincident with the increase in viral load that were not already present while viremia was suppressed, making antiviral drug resistance an unlikely explanation for the reemergence of viremia. While it is possible that secondary escape mutations arose subsequent to the R132T mutation and contributed to the return of viremia, none were identified in RT or Gag.

The reason for the late emergence of B27-KK10 escape mutations in the course of HIV-1 infection is not clear, but Kelleher et al. have hypothesized that selection for the more-common R132K escape mutation may require an antecedent L136M mutation in order to overcome structural constraints imposed by the p24 molecule (9). It is of interest to note that this putative "compensatory" L136M mutation was apparently not a structural prerequisite for the R132T escape mutation observed in patient TCH-043.

Viral escape in this patient was accompanied by a marked expansion in the number of CTL specificities targeted. It has been hypothesized that a broad, polyclonal CTL response may be advantageous for viral containment, an assumption which is supported by the survival advantage conferred by HLA class I heterozygosity (4). The temporal association of immunologic failure with the broadening of the CTL response in our patient suggests that the relationship between CTL breadth and immune control may be more complex than previously thought and that increased CTL breadth may, at least in some cases, be indicative of a failing immune response. Overall, the number of CTL specificities targeted by chronically HIV-1-infected adults does not correlate with viral load (1). A highly focused response targeting fewer epitopes has been found among long-term nonprogressors expressing HLA-B57 (12), although it could be argued that the narrow response in such subjects is related to decreased antigenic stimulation in the setting of minimal viral replication. The broadening of the CTL response in our patient cannot be readily attributed to an increase in plasma viral load, because the expansion in the number of targeted epitopes preceded the reemergence of measurable HIV-1 viremia. The observed association between CTL broadening and loss of immune control warrants further exploration in longitudinal studies.

REFERENCES

1. Addo, M. M., X. G. Yu, A. Rathod, D. Cohen, R. L. Eldridge, D. Strick, M. N. Johnston, C. Corcoran, A. G. Wurcel, C. A. Fitzpatrick, M. E. Feeney, W. R. Rodriguez, N. Basgoz, R. Draenert, D. R. Stone, C. Brander, P. J. R. Goulder, E. S. Rosenberg, M. Altfeld, and B. D. Walker. 2003. Comprehensive epitope analysis of human immunodeficiency virus type 1 (HIV-1)-specific T-cell responses directed against the entire expressed HIV-1 genome demonstrate broadly directed responses, but no correlation to viral load. J. Virol. 77:2081–2092.

2. Allen, T. M., D. H. O'Connor, P. Jing, J. L. Dzuris, B. R. Mothe, T. U. Vogel, E. Dunphy, M. E. Liebl, C. Emerson, N. Wilson, K. J. Kunstman, X. Wang, D. B. Allison, A. L. Hughes, R. C. Desrosiers, J. D. Altman, S. M. Wolinsky, A. Sette, and D. I. Watkins. 2000. Tat-specific cytotoxic T lymphocytes select for SIV escape variants during resolution of primary viraemia. Nature 407:386–390.

3. Barouch, D. H., J. Kunstman, M. J. Kuroda, J. E. Schmitz, S. Santra, F. W. Peyerl, G. R. Krivulka, K. Beaudry, M. A. Lifton, D. A. Gorgone, D. C. Montefiori, M. G. Lewis, S. M. Wolinsky, and N. L. Letvin. 2002. Eventual AIDS vaccine failure in a rhesus monkey by viral escape from cytotoxic T lymphocytes. Nature 415:335–339.

4. Carrington, M., G. W. Nelson, M. P. Martin, T. Kissner, D. Vlahov, J. J. Goedert, R. Kaslow, S. Buchbinder, K. Hoots, and S. J. O'Brien. 1999. HLA and HIV-1: heterozygote advantage and B*35-Cw*04 disadvantage. Science 283:1748–1752.

5. Feeney, M. E., K. A. Roosevelt, Y. Tang, K. J. Pfafferott, K. McIntosh, S. K. Burchett, C. Mao, B. D. Walker, and P. J. R. Goulder. 2003. Comprehensive screening reveals strong and broadly directed human immunodeficiency virus type 1-specific CD8 responses in perinatally infected children. J. Virol. 77:7492–7501.

6. Goulder, P. J., C. Brander, Y. Tang, C. Tremblay, R. A. Colbert, M. M. Addo, E. S. Rosenberg, T. Nguyen, R. Allen, A. Trocha, M. Altfeld, S. He, M. Bunce, R. Funkhouser, S. I. Pelton, S. K. Burchett, K. McIntosh, B. T. Korber, and B. D. Walker. 2001. Evolution and transmission of stable CTL escape mutations in HIV infection. Nature 412:334–338.

7. Goulder, P. J., R. E. Phillips, R. A. Colbert, S. McAdam, G. Ogg, M. A. Nowak, P. Giangrande, G. Luzzi, B. Morgan, A. Edwards, A. J. McMichael, and S. Rowland-Jones. 1997. Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. Nat. Med. 3:212–217.

8. Kaslow, R. A., M. Carrington, R. Apple, L. Park, A. Munoz, A. J. Saah, J. J. Goedert, C. Winkler, S. J. O'Brien, C. Rinaldo, R. Detels, W. Blattner, J. Phair, H. Erlich, and D. L. Mann. 1996. Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection. Nat. Med. 2:405–411.

9. Kelleher, A. D., C. Long, E. C. Holmes, R. L. Allen, J. Wilson, C. Conlon, C. Workman, S. Shaunak, K. Olson, P. Goulder, C. Brander, G. Ogg, J. S. Sullivan, W. Dyer, I. Jones, A. J. McMichael, S. Rowland-Jones, and R. E. Phillips. 2001. Clustered mutations in HIV-1 gag are consistently required for escape from HLA-B27-restricted cytotoxic T lymphocyte responses. J. Exp. Med. 193:375–386.

10. Klenerman, P., Y. Wu, and R. Phillips. 2002. HIV: current opinion in escapology. Curr. Opin. Microbiol. 5:408–413.

11. Martinez-Picado, J., M. P. DePasquale, N. Kartsonis, G. J. Hanna, J. Wong, D. Finzi, E. Rosenberg, H. F. Gunthard, L. Sutton, A. Savara, C. J. Petropoulos, N. Hellmann, B. D. Walker, D. D. Richman, R. Siliciano, and R. T. D'Aquila. 2000. Antiretroviral resistance during successful therapy of HIV type 1 infection. Proc. Natl. Acad. Sci. USA 97:10948–10953.

12. Migueles, S. A., M. S. Sabbaghian, W. L. Shupert, M. P. Bettinotti, F. M. Marincola, L. Martino, C. W. Hallahan, S. M. Selig, D. Schwartz, J. Sullivan, and M. Connors. 2000. HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. Proc. Natl. Acad. Sci. USA 97:2709–2714.

13. O'Brien, S. J., X. Gao, and M. Carrington. 2001. HLA and AIDS: a cautionary tale. Trends Mol. Med. 7:379–381.