Genetic and Stochastic Influences on the Interaction of Human Immunodeficiency Virus Type 1 and Cytotoxic T Lymphocytes in Identical Twins†

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Human immunodeficiency virus type 1 (HIV-1) evolves in vivo under selective pressure from CD8+ T-lymphocyte (CTL) responses, which are in turn determined by host and viral genetic factors, such as restricting major histocompatibility complex molecules and the available viral epitope sequences. However, CTL are derived stochastically through the random gene rearrangements to produce T-cell receptors (TCR), and the relative impact of genetic versus stochastic processes on CTL targeting of HIV and immune-driven viral evolution is unclear. Here we evaluate identical twins infected with HIV-1 as neonates from a common blood transfusion, with subsequently similar environmental exposures, thereby allowing controlled comparisons of CTL targeting and viral evolution. Seventeen years after infection, their CTL targeting of HIV-1 was remarkably similar. In contrast, their overall TCR profiles were highly dissimilar, and a dominant epitope was recognized by distinctly different TCR in each twin. Furthermore, their viral epitopes had diverged, and there was ongoing viral phylogenetic divergence between the twins between 12 and 17 years after infection. These results indicate that while CTL targeting is predominately genetically determined, stochastic influences render the interaction of HIV-1 and host immunity, and therefore viral escape and CTL efficacy, unpredictable.

The high mutation and replication rates of human immunodeficiency virus type 1 (HIV-1) are a substantial challenge to controlling its replication by pharmacologic or immunologic means. The lack of proofreading by the viral reverse transcriptase results in an error rate of nearly 1 per 104 bases incorporated (18). In the context of replication estimated at 1010 virions produced per day in an infected person (11, 29), it is likely that all potential point mutations are produced in vivo daily (20, 22). Thus, HIV-1 freely evolves in response to selective pressures (6, 25), which shape the predominating sequences in vivo.

In the setting of such fluid mutability, a major force shaping viral evolution in vivo is antiviral pressure applied by major histocompatibility complex class I (MHC I)-restricted HIV-1-specific CD8+ cytotoxic T lymphocytes (CTL). This arm of immunity is believed to have an important role in suppressing viremia; the development of the CTL response temporally correlates with the decline of viremia during primary infection (5, 16), and experimental in vivo CD8+ T-cell depletion in simian immunodeficiency virus (SIV)-infected macaques results in markedly increased viremia (13, 19, 26). Several studies in humans and macaques have demonstrated that mutations in CTL epitopes in vivo evolve to permit evasion of immunity (reviewed in reference 30); thus, CTL exert constraints on HIV-1 coding sequences in vivo. Associations of specific HIV-1 sequence polymorphisms with specific MHC I alleles across human populations (21) further confirm the central role of CTL in shaping HIV-1 evolution. The determinants of escape at the individual and cellular level, however, remain poorly defined (34).

Targeting of HIV-1 by CTL is also incompletely understood. The HIV-1 genome varies substantially, even between viruses within the same subtype, affecting the sequences of potential epitopes for CTL recognition. Furthermore, the CTL response in infected persons tends to target a small minority of the many potential epitopes defined by their MHC I haplotypes, and different persons with the same MHC I molecules usually target different epitopes restricted by those molecules (3). It is therefore unclear to what extent host genetic, viral genetic, or other factors contribute to this variability of viral targeting by CTL between infected individuals.

Unraveling the determinants of CTL targeting and evolution of HIV-1 sequences in vivo is therefore complicated by the
variability of viral sequences and host genetic factors. Here we evaluate identical twins who were perinatally infected from a common transmission source. This unfortunate circumstance allowed examination of CTL responses and viral evolution in a clinical setting, where host genetic factors and initial viral sequence are controlled.

MATERIALS AND METHODS

Human subjects. Subjects 1-05 and 1-06 are identical twins identified within a cohort of perinatally infected children in the Los Angeles area. Blood samples and clinical data were obtained after receiving approval from the institutional review boards of Children's Hospital Los Angeles and the University of California, Los Angeles.

MHC class I typing. High-resolution class I typing was performed by Pel-Freeze Biologicals.

Peripheral blood T-cell counts and viremia quantitation. Peripheral blood CD4\(^+\) and CD8\(^+\) T-lymphocyte subset measurements were determined by the clinical laboratory of Children's Hospital Los Angeles. Plasma HIV-1 RNA concentrations were determined by reverse transcription-PCR (RT-PCR; Roche Amplipcr HIV-1 Monitor).

Mapping of CD8\(^+\) T-cell responses against HIV-1. (i) Cells. Cryopreserved peripheral blood mononuclear cells (PBMC) were utilized to generate polyclonally expanded CD8\(^+\) T cells as previously described (33). Briefly, unfractionated PBMC were exposed to a CD3\(^4\) bisppecific antibody that preferentially expands CD8\(^+\) T cells, resulting in a polyclonal line of >95% CD3\(^+\)/CD8\(^+\) after 14 days of expansion (by flow cytometric analysis) (data not shown). (ii) IFN-γ ELISPOT. After 14 days, these cells were then screened for responses by gamma interferon (IFN-γ) ELISPOT using peptides from the NIH AIDS Research and Reference Reagent Repository as previously described (32). These peptides were 15-mers overlapping by 11 amino acids. The Pol (catalog no. 6446), Gag (no. 6445), and Rev (no. 6445) peptides were based on consensus B sequences from the Los Alamos National Laboratory HIV database. The Gag (no. 6869) and Env (no. 6451) peptides were based on HIV-1 DU422 and MN strain sequences, respectively. The initial screening was performed on pools of 16 or fewer peptides, and positive pools were then screened using 4-by-4 matrix pools as per the strategy described by Betts et al. (3). Candidate peptides identified by the matrices were then confirmed by individual testing by ELISPOT. Positive responses were defined as being at least two standard deviations above the mean for triplicate negative control wells.

T-cell receptor V\(_\beta\) spectratyping analysis. T-cell receptor V\(_\beta\) DCR3 length mapping was performed by RT-PCR as previously described (14). Briefly, CD8\(^+\) T cells were isolated from cryopreserved PBMC (12000 and 6001) by negative selection (Dynal, Great Neck, NY) as per the manufacturer’s protocol. TCR V\(_\beta\) transcripts were amplified by RT-PCR using a constant region primer, followed by gamma interferon (IFN-γ) ELISPOT using peptides from the NIH AIDS Research and Reference Reagent Repository as previously described (32). These peptides were 15-mers overlapping by 11 amino acids. The Pol (catalog no. 6208), Nef (no. 5189), Tat (no. 5136), Vpr (no. 6447), Vpu (no. 6444), Vif (no. 6446), and Rev (no. 6445) peptides were based on consensus B sequences from the Los Alamos National Laboratory HIV database. The Gag (no. 6869) and Env (no. 6451) peptides were based on HIV-1 DU422 and MN strain sequences, respectively. The initial screening was performed on pools of 16 or fewer peptides, and positive pools were then screened using 4-by-4 matrix pools as per the strategy described by Betts et al. (3). Candidate peptides identified by the matrices were then confirmed by individual testing by ELISPOT. Positive responses were defined as being at least two standard deviations above the mean for triplicate negative control wells.

RESULTS

Perinatally HIV-1-infected identical twins had highly similar environmental influences. In 1983, male identical twins, designated 1-05 and 1-06, were born at less than 32 weeks gestational age. During hospitalization in the neonatal intensive care unit, both were transfused with the same blood unit donated by an individual who subsequently tested positive for HIV-1 antibodies. At 7 years of age, both twins had lymphadenopathy, recurrent otitis media, asthma, and CD4\(^+\) T lymphopenia and were identified as HIV-1 seropositive. Starting at diagnosis, they were treated progressively with a variety of antiretroviral drug regimens, including single, double, and triple drug regimens, with poor adherence to treatment and persistent viremia. In 2000, when both twins had late-stage disease, they were placed on a combination regimen predicted to be effective by genotype testing (stavudine, lopinavir-ritonavir, and saquinavir). They were highly adherent to this regimen, resulting in sustained CD4\(^+\) T-lymphocyte and virologic responses (Fig. 1). Quantitation of peripheral blood T-cell receptor excision circles and naïve CD4\(^+\) T lymphocytes further demonstrated similar degrees of immune reconstitution accompanying this response to treatment (data not shown). Of note, the twins cohabitated and received all health care in parallel, including immunizations, up to the time of this study. Overall, starting from the time of diagnosis with late-stage infection, their clinical courses were nearly identical in the context of identical genetic backgrounds and similar environmental influences, including antiretroviral drug treatment, providing a unique opportunity to compare immune responses and viral evolution under controlled host milieu.

HIV-1-specific CTL targeting is similar between twins. Because CTL are an important factor in HIV-1 immunopatho-
genesis (28), the peptide reactivities of CTL responses in the twins were mapped and compared. These measurements were performed on cells from 12/00 and 6/01, 3 and 9 months after both twins had been started on effective combination antiretroviral therapy. The CTL responses detected in both twins predominately targeted Pol (Fig. 2). Twin 1-05 had detectable responses against 14 peptides (including 5 overlapping peptide pairs, indicating at least 9 recognized epitopes: 7 in Pol, 1 in Nef, and 1 in Tat), and Twin 1-06 had detectable responses against 4 peptides (including 1 overlapping peptide pair and at least 3 detected epitopes, all in Pol). The responses for twin 1-06, whose viremia was lower on treatment, were generally a subset of responses for twin 1-05. One of these shared peptide responses, targeting HKAIGTVLVGPTPVN (Pol 125-139; protease 69-83) was a consistently dominant response in both twins, while the other two shared peptide responses were inconsistently detected over time on treatment (data not shown). Because shared individual MHC I haplotypes usually do not predict CTL targeting in HIV-1-infected individuals (3), these data demonstrate that the HIV-1-specific CTL targeting is remarkably similar within the context of identical viral and host genetic backgrounds.

**CD8⁺ T-lymphocyte TCR profiles are dissimilar between the twins.** Interestingly, however, the targeted CTL epitopes differed between the twins (Fig. 2b). To compare in greater detail the CTL responses of the twins to HIV-1 infection, TCR Vβ spectratyping of the total CD8⁺ T-lymphocyte population was performed. The spectratype profiles of cryopreserved cells from 12/00 and 6/01 revealed that each twin had numerous TCR peaks deviating from the expected Gaussian size distribution of unexpanded populations within the 24 Vβ families tested, but that their patterns were grossly dissimilar (Fig. 3A; see also Fig. S1 in the supplemental material). While the profile within each twin remained significantly similar over these times (\( P < 0.01 \)), the spectratypes did not correlate between the twins (Fig. 3B) (Spearman rank coefficient, \(-0.19 \) to 0.30). Interestingly, the lack of correlation between twins was similar to HIV-1-infected unrelated subjects in general (based on comparisons between eight unrelated infected persons, mean of 0.28 ± 0.19 [data not shown]). These data indicated overall

**FIG. 1.** Clinical course and treatment of identical twins infected with HIV-1 via neonatal blood transfusion. Peripheral blood CD4⁺ T-lymphocyte counts (closed symbols) and plasma HIV-1 RNA levels (open symbols) are plotted for both twins (circles, twin 1–05; triangles, twin 1–06). The antiretroviral drug treatment history is indicated. ZDV, zidovudine; ddc, dideoxycytidine; ddl, didanosine; NVP, nevirapine; 3TC, lamivudine; NEL, nelfinavir; EFV, efavirenz; D4T, stavudine; SQV, saquinavir; LPV/r, lopinavir-ritonavir.

**FIG. 2.** HIV-1-specific CTL targeting of the twins. (a) The schematic indicates CTL targeting in the twins in 12/00 and 6/01. Upper and lower arrows indicate locations of recognized peptides for twins 1–05 and 1–06, respectively. (b) The screening clade B consensus peptides eliciting these responses by ELISpot are shown. The HIV-1 sequences in the twins (consensus of sequences obtained from PBMC) from 12/00 corresponding to recognized peptides or overlapping regions of consecutive peptides (containing the recognized epitope) are indicated. A dash indicates an amino acid identical to the clade B consensus sequence; x indicates lack of consensus among sequences.

<table>
<thead>
<tr>
<th>Recognized Peptide (Consensus B)</th>
<th>Location</th>
<th>Twin 1-05 ELISpot</th>
<th>Twin 1-06 ELISpot</th>
<th>Twin 1-05 Versus Consensus B Sequence</th>
<th>Twin 1-06 Versus Consensus B Sequence</th>
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<tr>
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dissimilarity of CD8⁺ T-lymphocyte expansions between the twins.

To assess whether individual CTL responses also differed, CD8⁺ T-lymphocyte spectratyping was performed on cultured PBMC, in the absence and presence of stimulation with the peptide (protease 69-83) that was dominantly targeted by CTL in both twins (ranging from about 20 to 80% of total HIV-1-specific CTL identified in repeated assays [data not shown]). Comparison of control and peptide-stimulated spectratypes allowed identification of peaks that specifically expanded upon stimulation (Fig. 4). Within the 24 Vβ families, each twin had multiple peaks that increased in response to the peptide, suggesting breadth of TCR recognizing this peptide (although we could not exclude nonspecific bystander expansions with this method). These expansions were entirely nonoverlapping across the 14 Vβ families for which data were available from both twins, further indicating that the TCR recognizing this peptide were distinct between the twins. In addition, among the 8 Vβ families for which expansion data were not available for twin 1-06, there were no peptide-specific expansions for twin 1-05, indicating that they had no shared responses in at least 22 of the 24 families. Overall, the spectratyping data revealed that despite similarity of recognized epitopes, the HIV-1-specific TCR were distinct between the twins, consistent with the stochastic nature of TCR generation.

HIV-1 sequence evolution is divergent between the twins. To evaluate HIV-1 genetic evolution in the twins, viral sequences were examined in blood samples from 1995 and 2000. Sequences in pol, env, and nef within each twin at each time point were separately phylogenetically clustered (Fig. 5). Within each twin, these genes exhibited divergence between 1995 and 2000, more in 1-05 than 1-06, indicating ongoing evolution between 12 and 17 years after infection. Sequence diversity at these time points was also greater within 1-05 than 1-06, con-
sistent with the greater rate of evolution (see Table S1 in the supplemental material). Comparing twins, the degree of divergence between sequences from 1-05 versus 1-06 was greater in 2000 than 1995, further indicating that the evolution was continuing to progress in distinct directions. Further sequences in gag and vpr from 2000 were also analyzed and also demonstrated dissimilarity between the twins (see Fig. S2 in the supplemental material). In view of prior data suggesting that HIV-1 infection by blood transfusion transmits a large viral inoculum with comparable quasispecies distribution (9), these findings strongly imply that HIV-1 diverged considerably between the twins by 12 years after infection and was continuing to diverge.

**DISCUSSION**

Sterilizing immunity against HIV-1 is considered an unlikely, and possibly unattainable, goal for vaccine development, but protection against disease may be possible if persistently effective immune responses can be generated. Because CTL are important in the immune containment of HIV-1, many vaccine development efforts have emphasized generating broad and intense CTL responses (31). Experimental vaccination of macaques against SIV has provided support for this concept but also demonstrated that viral escape from vaccine-induced CTL responses can result in vaccine failure (2). It is thus likely that interactions between HIV-1 and CTL are crucial in the ability of vaccines to provide durable suppression of viremia and prevent disease.

The subjects of our study provide an unfortunate but unique opportunity to compare CTL responses and HIV-1 evolution in a setting where host genetic factors and initial viral sequences are controlled in two individuals. CTL targeting of HIV-1 by these twins is remarkably similar, shedding light on this poorly understood process. In contrast to other viral infections, few clear patterns of true immunodominance have emerged for HIV-1 (3), and most infected individuals sharing a common MHC I allele do not target the same subset of potential epitopes restricted by that allele (7). The present study finds that when the host and viral genetic factors are held constant, the targeting of HIV-1-specific CTL is then similar. This suggests that epitope targeting is primarily determined by

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**FIG. 4.** Peptide-specific T-cell receptor $\beta$ spectratype analysis. PBMC from 9/01 were cultured in the presence or absence of the Pol peptide HKAIGTVLVGPTPVN (Pol 125–139; protease 69–83), which was the dominantly recognized peptide for both twins (accounting for approximately 50% of the detected HIV-1-specific CTL [data not shown]). Spectratyping then was performed on the CD8+ cells from each twin. Histograms from four representative $\beta$ families are shown; peptide-stimulated expansions are shaded (peaks whose ratio versus the median of all peaks in the family increased by at least 2 and rose at least twofold after stimulation). The accompanying table indicates the specific peaks within each of the 24 $\beta$ families that were expanded in response to peptide stimulation. N.D. indicates that data were not available.
host and/or pathogen genetics and not particularly subject to stochastic influences. CTL targeting differences between persons sharing common MHC I alleles is therefore likely to be determined primarily by differences in infecting HIV-1 genotype or the influence of additional host genetic factors, such as competing HLA alleles or polymorphisms in antigen processing factors.

T-cell receptor generation, however, is a stochastic process. Epitope-specific CTL responses within individuals tend to be comprised of multiple clones with differing TCR (10). In contrast to their shared targeting, the spectratype profiles of the twins are strikingly dissimilar, and the dominant detected CTL response is comprised of multiple clones that are clearly distinct between twins. Thus, despite phenotypic similarities in targeting, the responding CTL differ at the TCR molecular level. Because different TCR recognizing the same epitope vary in functional properties, such as ligand avidity and recognition of different epitope escape variants (34), important aspects of CTL function are stochastically determined, as reflected by divergent viral sequence evolution in the twins. Our observations are consistent with the findings of Biggar et al., who studied HIV-1 evolution following mother-to-child transmission for 14 fraternal and 5 identical twins (4). Although the period of follow-up was relatively brief (12 to 16 weeks on average), HIV-1 sequences diverged between twins, and there did not appear to be a difference in the divergence rates between identical versus fraternal twins.

In a landmark study, Moore et al. demonstrated significant associations of MHC I alleles with specific HIV-1 sequence polymorphisms across a population of infected persons (21). These data suggest that among persons sharing certain MHC I alleles, there are generalized patterns of epitope targeting and escape mutations across populations. Less clear is the extent to which MHC I phenotype predicts targeting and escape at the level of the individual. Our results suggest that interactions of CTL and HIV-1 are not consistent at the level of the individual, due to the stochastic differences of TCR, despite the existence of some shared escape patterns across populations (as observed by Moore et al.). Thus, there is great variability in CTL escape within individuals due to stochastic factors.
The differences in targeted epitope sequences are consistent with stochastic TCR variability, given the tendency of different TCR targeting the same epitope to vary in recognition of epitope variants (34). A caveat to this finding are the differences in the early antiretroviral treatment regimens of the twins, which could have affected Pol sequences and the CTL targeting them. However, the twins had been on the same treatment regimens for 2 years at the time of sequencing. Additionally, two of the three observed protease sequence differences between the twins in amino acids 69 to 83 were not attributable to known protease inhibitor resistance mutations, and none of the drugs would be expected to affect the integrase region targeted by both twins, which also varied in sequence.

Moreover, the presence of a greater degree of diversity and progressive divergence in one twin reflects the unpredictability of immune pressure on HIV-1 despite genetically identical backgrounds and similar immunological targeting. While the twins had similar clinical and immunologic parameters from the time of diagnosis and treatment, it is unknown whether they had the same rate of disease progression before development of AIDS (1983 to 1990). However, because different mutations were selected by CTL in each twin, it seems possible that viral fitness costs for escape could vary significantly between twins and, thus, that the efficacy or durability of CTL containment could also vary.

In summary, our results illustrate the importance of stochastic influences in the interaction of the cellular immune response and HIV-1 despite the genetic determination of CTL targeting and variability in the constraints imposed by immunity. It is unclear whether vaccination approaches can yield cellular immune responses that will interact with HIV-1 in a predictable manner. For the production of a CTL-based vaccine to prevent disease but not infection, this may be a substantial barrier to consistent efficacy even when viral sequences and host genetics are considered in the vaccine design.

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