Measurement of HIV-1 CRF02_AG–Specific T Cell Responses Indicates the Dominance of a p24\textsuperscript{gag} Epitope in Blood Donors in Abidjan, Côte d’Ivoire

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Studies of HIV infection in the developing world are important, because it is where >95% of new HIV infections occur. Although current HIV-1 vaccine candidates are not likely to provide sterilizing immunity, they have the potential to slow progression to AIDS and to reduce viral loads, thereby contributing to reduced virus transmission between individuals and in the population as a whole. The efficacy that a hypothetical universal HIV-1 vaccine would have remains unknown, and many researchers are focused on generating regional vaccines containing HIV-1 genes from a single subtype.

Because virus-specific CD8+ T cells have been implicated in the initial control of viremia in HIV-1–infected humans and simian immunodeficiency virus–infected macaques (reviewed in [3]), the goal in the development of many candidate HIV vaccines is to induce high levels of these cells before infection. One measure of HIV-specific T cell responses involves the enumeration of interferon (IFN)–γ–secreting T cells by use of the enzyme-linked immunosorbent assay (ELISPOT) assay. Recently, the ELISPOT assay has been used to identify T cell epitopes in non–subtype B viruses (summarized in [4]) that predominate in areas such as sub-Saharan Africa, where the global HIV epidemic is concentrated and where extensive studies of T cell responses in the context of the regional HLA genetic background have not previously been conducted. This identification of viral epitopes that elicit T cell responses in HIV-infected persons is important for characterizing cellular immune responses directed against a particular virus subtype, for designing vaccine candidates, and for preparing for vaccine evaluation.

Here we present the first evaluation of T cell responses directed against HIV-1 CRF02_AG epitopes in the Ivorian population, by use of gag and pol consensus sequences for peptide design and the ELISPOT assay. This assessment of HIV-1 CRF02_AG–specific T cell responses elicited by natural infec-
tion was conducted in preparation for clinical trials of a multigene HIV-1 CRF02_AG DNA vaccine that has been developed for West Africa and West-Central Africa [5].

**Donors, materials, and methods.** Forty-two HIV-1– or HIV-1/2–seropositive and 14 HIV-seronegative blood samples were obtained from the Centre National de Transfusion Sanguine, Abidjan, Côte d’Ivoire. Informed consent for blood donation was given by all study subjects. All samples were anonymized before testing; thus, this study was deemed to be exempt from full review by the Institutional Review Board of the Centers for Disease Control and Prevention (CDC) and the ethical committee of the Ministry of Health, Côte d’Ivoire. Because use of antiretroviral therapy (ART) was rare in Côte d’Ivoire at the time the present study was conducted, we considered the donors to be ART naive.

Whole blood was collected into 1-U bags containing EDTA. Plasma and peripheral-blood mononuclear cells (PBMCs) were isolated by use of lymphocyte separation medium (MP Biomedicals). PBMCs either were used fresh or were cryopreserved in fetal bovine serum containing 10% dimethyl sulfoxide (DMSO). CD4+ T cells were counted by standard flow cytometry with a FACScan cytometer, fluorochrome-conjugated monoclonal antibodies, and CellQuest software (Becton Dickinson).

HIV serostatus was determined by an ELISA-based parallel testing algorithm [6]. Plasma HIV-1 load was quantified by the Amplicor HIV-1 Monitor Test (version 1.5; Roche Diagnostics), which accurately quantifies subtype AG recombinant viruses [7].

Class I HLA intermediate/high-resolution molecular typing was performed by sequence-specific primer polymerase chain reaction (PCR) on DNA extracted from frozen PBMCs at the Laboratories at Bonfils (Denver, CO). High-resolution class I HLA typing was performed by sequence-specific oligonucleotide probe hybridization techniques [8]. Nonresolved ambiguities are reported as 2-digit denominations.

Peptide sequences were based on partial gag p24 (aa 25–172) and pol protease (aa 8–92) HIV-1 CRF02_AG consensus sequences. The p24 consensus sequence has been described elsewhere [2]. The protease consensus sequence was derived from 29 protease inhibitor–naïve specimens collected in 1988 (n = 3) and 1998 (n = 26) in Côte d’Ivoire. Twenty-nine p24**agg** and 15 protease peptides, each 15 aa long and overlapping by 10 aa, were synthesized at the Biotechnology Core Facility, Scientific Resources Program, National Center for Infectious Diseases, CDC. Pools of 5 peptides were tested in two 5-by-5 pool matrices, such that each pool had one peptide in common with one other pool in its matrix. Seven 9-aa-long peptides overlapping by 8 aa were synthesized from EGATPQDLNMMLNIV (aa 45–59). Peptides were reconstituted in 100% DMSO, to a concentration of 100 mg/mL.

ELISPOT assays were performed as described elsewhere [9]. In brief, 2 × 10^6 PBMCs/well were added in duplicate. The final concentration of each peptide was 10 μmol/L. Plates were incubated either for 36 h (15-aa-long peptides) or for 16 h (9-aa-long peptides), to allow for optimal antigen processing. Phytohemagglutinin (PHA; 5 μg/mL) and DMSO (0.1%) were used as positive and negative controls, respectively. Spots were counted by eye with an inverted stereomicroscope (Olympus SZ-ST). Specific spot forming cell (sfc) values were calculated by subtracting the mean of negative control wells. Responses were considered to be significant when they were both more than twice the mean of the negative control well and >50 sfc/1 × 10^6 PBMCs.

Correlation analysis was performed with the nonparametric Spearman’s rank correlation test, by use of Prism software (version 4.0a; Applied Biosystems). P values were 2-tailed; significance was set at .05.

**Results.** Of the 40 HIV-1– and 2 HIV-1/2–infected donors, 34 (81%) responded to at least 2 peptide pools. Of the 14 HIV-uninfected donors, none responded to any peptide pool (data not shown).

We examined the impact of CD4+ T cell depletion and plasma viral load on the magnitude and breadth of IFN-γ ELISPOT responses. In the HIV-infected donors, the median absolute CD4+ T cell count was 515 cells/μL (range, 50–986 cells/μL), and the median plasma viral load was 4.5 log_{10} RNA copies/mL (range, from below the limit of detection to 6.7 log_{10} RNA copies/mL). Plasma viral load, but not absolute CD4+ T cell count, correlated inversely with the magnitude (P < .02; figure 1A) and breadth (P < .05; figure 1B) of the IFN-γ ELISPOT response.

The most frequently recognized peptide pool was pool 6 (containing only p24**agg** peptides), which elicited a response from 32 (76%) of the 42 HIV-infected donors (figure 2A). Pool 3 (containing p24**agg** and protease peptides) was the next most frequent inducer of IFN-γ and was recognized by 22 (52%) of the 42 of HIV-infected donors (figure 2A). All pool 3 responders also responded to pool 6, indicating that the shared p24**agg** peptide (EGATPQDLNMMNIV) was likely recognized by these donors. To confirm EGATPQDLNMMNIV reactivity, IFN-γ ELISPOT assays were performed on a second sample from 17 responders with available frozen PBMCs. Of these samples, 13 (76%) demonstrated significant IFN-γ ELISPOT responses when stimulated with EGATPQDLNMMNIV peptide alone (figure 2B). Samples from 4 (24%) of the donors (IC129, IC150, IC154, and IC159) did not meet the criteria for a positive response.

Because EGATPQDLNMMNIV was recognized by many donors, we sought to identify the optimal epitope within this 15mer. Intracellular cytokine staining and proliferation assays with CD8+ T cell–depleted PBMCs indicated that responses to EGATPQDLNMMNIV were mediated by CD8+, and not CD4+, T cells (data not shown). Thus, to map the minimal epitope, we synthesized seven 9-aa-long peptides overlapping by 8 aa that spanned EGATPQDLNMMNIV (figure 2C, at
Figure 1. HIV-1 CRF02_AG–specific T cell responses correlate with plasma viral load, but not CD4+ T cell count, in HIV-1– and HIV-1/2–infected blood donors in Côte d’Ivoire. The magnitude (panels A and C) and breadth (panels B and D) of the interferon-γ enzyme-linked immunospot responses were compared with either plasma viral loads (panels A and B; n = 42) or absolute CD4+ T cell counts (panels C and D; n = 27). Twenty peptide pools were analyzed for magnitude determinations, and 10 peptide pools were analyzed for breadth determinations. Correlation analysis was performed with the nonparametric Spearman’s rank correlation test. NS, nonsignificant; PBMCs, peripheral-blood mononuclear cells; sfc, spot-forming cell.

right). IFN-γ ELISPOT assays were performed on available frozen PBMCs from 11 EGATPQDLNMMLNIV responders. All 11 donors responded to a central nonamer (TPQDLNMML [TL-9]; figure 2C) that is a known cytotoxic T lymphocyte (CTL) epitope [4]. The magnitude of the response to TL-9 was higher than that to EGATPQDLNMMLNIV in 10 (91%) of the 11 donors (figure 2B and 2C), indicating that TL-9 was the optimal CTL epitope in these donors. All but 1 of the TL-9 responders were found to carry an HLA allele previously demonstrated to present the HIV-1 subtype A (HLA-B53) or subtypes B/C/D (HLA-B*0702, -B*4201, -B*8101, and -Cw*0802) variant of the TL-9 epitope [4]. HLA-B*5301, which represents the most prevalent HLA-B allele in Côte d’Ivoire [10], was present in 6 (55%) of the 11 TL-9 responders (figure 2C). In 1 donor (IC127), responses to PQDLNMMLN and DLNMMLNIV were of greater magnitude than the response to TL-9.

Discussion. This report presents the first description of HIV-1 CRF02_AG–specific T cell responses in individuals in Côte d’Ivoire. A candidate DNA vaccine for West Africa and West-Central Africa that contains CRF02_AG gag, pol, and env genes has been developed [5], and our study was conducted in preparation for clinical trials in this region. For vaccine trials in developing countries to be as effective as possible, it should be feasible to implement the methods used (e.g., the ELISPOT assay) to evaluate candidate vaccines in regional laboratories.

Using consensus sequences as probes, we identified a p24ag peptide, TL-9, in CRF02_AG that was recognized by many HIV-1– and HIV-1/2–infected blood donors.

Obtaining measurements of HIV-specific T cell responses in the Ivorian population is important, because few studies of HIV-infected individuals in Côte d’Ivoire have been conducted to date, and only subtype B reagents have been used. CRF02_AG differs significantly from subtype B, and the MHC genotypes in Côte d’Ivoire differ from those of better-studied regions. Via sequencing, we confirmed that the HIV-1 env of most of the donors in the present study clustered with CRF02_AG reference strains (data not shown). We next found an inverse relationship between plasma viral load and both the magnitude and breadth of HIV-1–specific T cell responses, which is consistent with the findings of one previous report [11] but not of another [12]. This study-specific identification of an association (or lack thereof) between the immune response and plasma viremia is likely dependent on the specific gene products used, the stage of infection of the study participants, and/or the source virus used in peptide design.

We focused on the Gag and Pol proteins, because they are among the more highly conserved regions of HIV-1. In CRF02_AG, gag derives largely from subtype A, and the protease region of pol derives from subtype G [13]; thus, we studied T cell responses to regions representing the intersubtype re-
Figure 2. Frequent recognition of the peptide EGATPQDLNMMLNIV in HIV-1- and HIV-1/2-infected blood donors in Côte d’Ivoire and identification of the optimal epitope, TPQDLNMML. **A,** Peptide screening. Two peptide pool matrices (one comprised of pools 1–10, and another comprised of pools 11–20) were used to screen for p24<sub>gag</sub> and Pol protease 15mer peptides that elicited HIV-1 CRF02_AG–specific interferon (IFN)–γ secretion by T cells in an enzyme-linked immunospot (ELISPOT) assay. The amino acid sequence of the shared peptide from the 2 most frequently recognized pools (pools 6 and 3) is indicated. **B,** ELISPOT assay with shared peptide. The IFN-γ ELISPOT assay was repeated for 17 of the 22 HIV-1– and HIV-1/2–infected subjects who recognized peptide pools 3 and 6 together, with the shared peptide EGATPQDLNMMLNIV used to stimulate peripheral-blood mononuclear cells (PBMCs). The dashed line drawn at 50 spot-forming cells (sfc)/PBMCs indicates one of the criteria used to determine a positive response. **C,** Determination of optimal epitope. Seven 9-aa-long peptides overlapping by 8 aa (shown at right) were synthesized, to determine the optimal epitope contained within the EGATPQDLNMMLNIV 15mer. IFN-γ ELISPOT assays were then performed for 11 EGATPQDLNMMLNIV responders; responses are shown for the control (10% dimethyl sulfoxide [DMSO]); the optimal nonamer, TPQDLNMML; and the other nonamers analyzed. Donors are indicated along with their HLA type.

combination of CRF02_AG. Use of a consensus sequence as the reference strain for peptide synthesis, as was done in the present study, has become more widely accepted as the limitations of single viral isolates become apparent [14], although studies of more variable regions of the HIV-1 genome may benefit from the use of peptides based on autologous sequences. We observed a mean identity of 94.6% for p24<sub>gag</sub> and of 93.2% for protease between consensus and autologous sequences (data not shown), illustrating the usefulness of the consensus sequence for the detection of T cell responses to these regions.

The most frequent T cell response in the present study population was against peptide pool 6. The region of p24<sub>gag</sub> covered by pool 6 is dense with CTL epitopes, with fewer and less-well-defined CD4 epitopes [4]. We did not consistently detect T cell
responses against protease, perhaps because it contains fewer known CTL epitopes than do other regions of pol (such as reverse transcriptase and integrase) and because the majority of these epitopes are restricted by members of the HLA-A2 supertype [4], which are infrequently found in African populations. The EGATPQDLNMMLNIV peptide was the most frequently recognized region of the p24⁴⁰ consensus, and, within EGATPQDLNMMLNIV, we identified TL-9 as the optimal epitope in 91% of the donors assayed. This nonamer has been characterized as an HLA-B53–restricted epitope in HIV-1 subtype A–infected Gambians [15]. The 10 donors in our study who had optimal responses to TL-9 carried HLA alleles that restrict either the HIV-1 subtype A or subtypes B/ C/D variant TL-9 peptide [4], with the majority (6/10) being positive for HLA-B*5301. However, because we did not study single MHC–expressing antigen-presenting cells and T cell lines/clones, we cannot definitively confirm the elements restricting EGATPQDLNMMLNIV or TL-9 in our Ivorian donors. Other limitations of the present work include the fact that the donors were not well characterized with respect to infection history or duration and that we focused on small regions of the HIV genome. Although it is likely that there are immunodominant peptides in other proteins of HIV-1 CRF02_AG and in Gag or Pol for individuals with HLA types not studied here, the high frequency of responders to p24⁴⁰, and particularly to EGATPQDLNMMLNIV or TL-9, suggests that further studies of this epitope and of any association between it and viral control may be important for the development of epitope-based vaccines for Côte d’Ivoire. Although it is difficult to develop candidate HIV-1 vaccines in part because of viral inter- and intrasubtype variability, epidemiologic surveys of host immune responses directed against endemic viral sequences will likely contribute to the next generation of HIV-1 vaccines.

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References