Potentially Exposed but Uninfected Individuals Produce Cytotoxic and Polyfunctional Human Immunodeficiency Virus Type 1-Specific CD8+ T-Cell Responses Which Can Be Defined to the Epitope Level

A. L. Erickson, C. B. Willberg, V. McMahan, A. Liu, S. P. Buchbinder, L. A. Grohskopf, R. M. Grant, and D. F. Nixon

Division of Experimental Medicine, Department of Medicine, UCSF, San Francisco, California 94110; Gladstone Institute of Virology and Immunology, UCSF, San Francisco, California 94158; Division of VCF and Epidemiology, Department of Medicine, UCSF, San Francisco, California 94143; and Epidemiology Branch, Division of HIV/AIDS Prevention, Centers for Disease Control and Prevention, Atlanta, Georgia 30333

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We measured CD8+ T-cell responses in 12 potentially exposed but uninfected men who have sex with men by using cytokine flow cytometry. Four of the individuals screened exhibited polyfunctional immune responses to human immunodeficiency virus type 1 Gag or Vif. The minimum cytotoxic T lymphocyte epitope was mapped in one Gag responder.

Understanding the specific immune responses conferring protection from infection in individuals exposed to human immunodeficiency virus type 1 (HIV-1) is critical for vaccine design. Immune parameters (CD4+ T-helper responses, cytotoxic T lymphocytes [CTLs], and soluble factors, including cytokines, B-chemokines, CD8+ toxic T lymphocytes [CTLs], and soluble factors, including cytokines, B-chemokines, CD8+ T-cell responses, and anti-HIV immunoglobulin A antibodies) have been detected in HIV-exposed but HIV-uninfected individuals at variable levels (5, 10–14, 16–18, 20, 22–24, 26, 27, 29–31). Not all studies have detected such responses, fostering controversy as to the technical assessment and validity of these results. CTL studies of HIV-exposed, uninfected individuals are challenging, often demonstrating weak antigen-specific responses that are neither readily mappable to epitopic determinants nor consistently detectable over time (1, 15, 19). There have been no convincing demonstrations of the fine mapping of an epitope recognized by a CTL line in individuals exposed but uninfected. We describe the detection of cytotoxic CD8+ T-cell immune responses to HIV-1 Gag or Vif antigens by cytokine flow cytometry (CFC) in a cohort of potentially exposed but uninfected men who have sex with men (MSM). Fine mapping revealed reproducible responses to a single epitope in one individual. Moreover, CTL lines were generated validating the responses generated in the CFC assay, providing the first convincing evidence for antigenic specificity of a CD8+ T-cell response in a potentially exposed but uninfected individual. CD8+ T-cell immune responses were analyzed in 12 MSM enrolled in a clinical safety trial of oral antiretroviral preexposure prophylaxis. None had yet received study medication. Entry criteria required at least one high-risk sexual exposure in the last 12 months. Thirteen (19%) of the 679 men screened tested HIV-1 positive at screening or enrollment. Among the 400 men enrolled in the cohort, 56% reported unprotected anal sex within the previous 3 months and 29% with an HIV-positive or unknown-status partner. Oraquick anti-HIV-1 antibody testing using oral fluid was performed at each study visit, and peripheral blood mononuclear cells (PBMC) were banked at enrollment, at 1 month postenrollment, and at 3-month intervals thereafter. PBMCs were thawed and restimulated with 5 μg/ml HIV consensus B pooled peptides (Nef, Tat, and Vif) and HXB2 Gag pool peptides (NIH AIDS Research and Reference Reagent program) or Staphylococcus enterotoxin B (SEB) (1 μg/ml; positive control) for 12 to 16 h. Brefeldin A and monensin were added for the last 5 h. PBMCs were then stained with extracellular antibodies (CD8 and an amine dye live/dead cell marker) and intracellular antibodies (gamma interferon [IFN-γ], tumor necrosis factor alpha [TNF-α], and CD3). Because it is believed that polyfunctional CD8+ T cells play an important role in the control of HIV-1 infection (6), and it has been shown that CD8+ T cells expressing TNF-α and IFN-γ are representative of a cytotoxic T-cell phenotype (21), CFC was performed, gating on the CD3+ CD8+ TNF-α+ and/or IFN-γ+ cells. Of the 12 individuals screened by CFC, four had detectable Gag (n = 3) or Vif (n = 1) responses (Fig. 1a to c). Roche ultralow Cobas 1.5 HIV-1 RNA viral load testing with a lower limit of detection of 2.5 copies/ml was performed on plasma from the Gag and Vif responders and found to be negative at all time points studied. Longitudinal analysis over 6 months of the Vif-responding individual initially revealed a clear response. Two months of follow-up failed to detect a Vif response; however, 3 months later, the Vif response had returned (Fig. 2). It is possible that this individual was exposed to HIV-1 prior to the first determination and again prior to the third determination. It is interesting to note that the response at the third time point exceeded the first, consistent with a recall response. PBMC from the four responding individuals were restimulated with peptide pools that previously elicited responses,
using samples from the same time point as in the CFC assay. Antigen-specific populations were expanded and measured via standard chromium ($^{51}$Cr) release assay (9). Among these individuals, only the CD8$^+$ HLA$^A$11001 T cells from one Gag responder showed detectable lysis by 51Cr release assay (Fig. 3). The Gag-specific cell line lysed the P55 Gag peptide pool target used to restimulate the PBMC as well as the P17 Gag pool target and a subset of the P17 pool, called P17 pool 1 (containing HXB2 Gag peptides 4985 to 4994, representing amino acids (aa) 1 to 51 of the P17 protein of Gag) (Fig. 3a). To identify the 15-mer peptide being targeted by the Gag-specific cell line, pool 1 of P17 was broken down into the 10 individual peptides (4985 to 4994, representing aa 1 to 51 of Gag P17) comprising the pool (Fig. 3b). The Gag-specific cell line recognized the 15-mer peptide EKIRLRPGGKKKYKL (p4989; P17 aa 17 to 31) and not any of the other peptides in pool 1 of P17 Gag. This line also lysed a recombinant vaccinia Gag-infected target but not a control recombinant vaccinia Env target (Fig. 3c), indicating that it was capable of recognizing

FIG. 2. Cytokine flow cytometric assays were performed on PBMC from four longitudinal time points from an exposed but uninfected individual who responded to Vif. PBMC were restimulated with medium only (unfilled bar), a Vif peptide pool (black bar), or SEB (horizontally striped bar) for 16 h. Samples were gated on CD3$^+$ CD8$^+$ lymphocytes and analyzed for the expression of IFN-γ and TNF-α. Background cytokine cutoff levels were determined by including fluorescence-minus-one and PBMC controls for each individual in the CFC assay. Additionally, PBMC from a blood bank donor were stimulated with the same panel of antigens and run in parallel in each assay as a means of providing intra-assay control and to aid in the determination of background cytokine cutoff levels. Panels a and b represent dot plots of an individual Gag responder and Vif responder, respectively. In panel c, all subjects responded to the SEB positive control. However, only 4 of the 12 subjects produced detectable HIV-1 responses (3 responded to Gag and 1 to Vif). Results are expressed as the percentages of CD3$^+$ CD8$^+$ T cells expressing IFN-γ and TNF-α.

FIG. 1. Cryopreserved PBMC from 12 HIV-exposed uninfected MSM were thawed and stimulated for 16 to 18 h with HIV-1 clade B 15-mer peptides spanning the Gag (123 peptides), Nef (49 peptides), Tat (23 peptides), and Vif (46 peptides) antigens at 5 μg/ml final concentrations. SEB (1 μg/ml) served as a positive control antigen. Responses were assessed by CFC, gating on CD3$^+$ CD8$^+$ lymphocytes, and analyzed for IFN-γ and TNF-α expression. Background cytokine cutoff levels were determined by including fluorescence-minus-one and PBMC controls for each individual in the CFC assay. Additionally, PBMC from a blood bank donor were stimulated with the same panel of antigens and run in parallel in each assay as a means of providing intra-assay control and to aid in the determination of background cytokine cutoff levels. Panels a and b represent dot plots of an individual Gag responder and Vif responder, respectively. In panel c, all subjects responded to the SEB positive control. However, only 4 of the 12 subjects produced detectable HIV-1 responses (3 responded to Gag and 1 to Vif). Results are expressed as the percentages of CD3$^+$ CD8$^+$ T cells expressing IFN-γ and TNF-α. EU, exposed but uninfected.
endogenously synthesized antigen. Therefore, it is likely that the observed antigen specificity was not an artifact of peptide in vitro restimulation. Interestingly, this is an epitope-rich area in Gag, containing two overlapping HLA-A*0301-restricted epitopes, KIRLRPGGK (KK9; P17 aa 18 to 26) and RLRPGGKKK (KR9; P17 aa 20 to 28), both present in the P17 peptide from aa 17 to 31. HLA typing by single specific primer PCR confirmed this individual was HLA-A*0301 positive. As the KK9 epitope was also present within a second, overlapping peptide (P17 aa 13 to 27) not recognized by the Gag specific line, it was probable that KR9 was the true epitope. This was confirmed by comparing the ability of the Gag cell line to recognize and kill target cells presenting either the minimal KR9 epitope or the P17 peptide from aa 17 to 31 (containing both epitopes) (Fig. 4). The Gag cell line lysed both targets with equal efficiency, indicating that the A*0301-restricted KR9 epitope was the epitope targeted by the exposed but uninfected individual, who remained HIV-seronegative at this time point. Notably, this epitope has been previously identified to be targeted in acute infection. Moreover, KR9 is considered an immunodominant epitope (3, 4, 7, 32).

We have shown that a subset of potentially exposed but uninfected MSM have detectable Gag and Vif CTL responses by CFC assay and have validated this method of immune detection by mapping the HLA-A*0301-restricted P17 Gag CTL epitope KR9 in one Gag responder. The detection and fine mapping of a T-cell epitope in an exposed but uninfected individual is an important advance in confirming the validity of responses seen at a low magnitude in exposed but uninfected individuals. The epitope mapped here is restricted by HLA-A*0301 and is identical to one detected and previously described in HIV-1-infected subjects. The variability of the responses seen in these subjects over time suggests that the T cells are reactive to low-level exposure that does not result in an infection or sustainable response. It cannot be ruled out in the present study that there was not a low-level infection, since in one study of exposed but uninfected individuals, it was shown that very low levels of virus could be PCR amplified and sequenced from two individuals that were antibody negative (33). However, RNA viral-load testing with a lower limit of detection of 2.5 copies/ml was found to be negative at all time points studied within our population. Whether the T cells generated at one time point are then involved in protection against infection at a subsequent exposure is unknown (2, 25).

We have demonstrated that individuals potentially exposed to HIV-1 but uninfected can make functionally competent CD8+ T-cell responses. Moreover, these responses display polyfunctionality and are capable of killing targets presenting cognate epitopes. It may be speculated that the generation of strong polyfunctional CD8+ T-cell responses might confer protection against HIV-1 infection, suggesting that the develop-
ment of a CDS+ cell-based vaccine may be feasible. However, as this work is limited in having performed fine mapping in only one individual, it will be important to further compare individuals who remain uninfected despite high-risk activities to those who subsequently seroconvert.

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