

Antibody Responses to HIV-1 gp120 Hypervariable Regions in Six Long-Term Non-Progressors

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Abstract: Our long-term goal is to discover the combination of host parameters that help some HIV-infected individuals to resist progression to AIDS. In this study, we examined antibody responses using multiple samples obtained from a cohort of Long-Term Non-Progressors (LTNPs). Our hypothesis is that antibody responses to variable regions of the HIV-1 envelope glycoprotein are involved in reducing the viral load associated with LTNPs and that these specific immune responses influence susceptibility to disease progression. Multiple plasma samples were obtained from patients identified as LTNPs with the objective of characterizing humoral immune response directed to the five hypervariable regions of the envelope glycoprotein. Antibody binding was tested against peptides representing the five hypervariable regions of gp120, as well as against analog peptides representing different isolates of HIV-1 and against recombinant envelope glycoprotein. LTNPs have specific antibodies to the hypervariable regions of the envelope glycoprotein and develop different patterns of antibody recognition to variable epitopes of envelope glycoprotein. These antibodies can be detected using HIV peptides as capture antigens.

Keywords: HIV-1, Envelope, Peptide, Long-Term Non-Progressors, Humoral Immune Response

Introduction

The envelope glycoprotein (Env) of HIV-1 is formed by heterodimers in a trimeric configuration, consisting of three gp120 surface envelope glycoproteins non-covalently bound to three gp41 transmembrane glycoproteins (Helseth *et al.*, 1991; Wyatt and Sodroski, 1998). Each gp120 monomer contains five hypervariable regions (V1-V5) separated by five constant regions (C1-C4) (Modrow *et al.*, 1987). The gp120 surface envelope glycoprotein of HIV-1 mediates viral attachment to target cells and elicits antibodies that neutralize HIV infection (Pantophlet and Burton, 2006). When gp120 binds to the cellular CD4 receptor, it undergoes conformational changes that expose the binding site of gp120 for chemokine co-receptors, including CCR5 or CXCR4 (Feng *et al.*, 1996).

The third hypervariable region (V3) is considered the major viral determinant of co-receptor specificity due to the fact that the co-receptor binding site resides primarily in the V3 region following conformational changes induced by binding to CD4. However, it is clear that V1, V2 and other regions are also involved (Speck *et al.*, 1997; Kwong *et al.*, 1998; Jensen and van't Wout, 2003). The V3 region also contains antigenic epitopes that induce both cellular and humoral immune responses, including neutralizing antibodies (Fomsgaard *et al.*, 1998; Cardozo *et al.*, 2009; Swetnam *et al.*, 2010).

The V1 and V2 regions are also highly variable in polymorphism, genetic sequence and N-linked glycosylation sites, factors that may affect attachment of gp120 to target cells, the usage of co-receptors and recognition by neutralizing antibodies (Hughes and Bell,

1997; Curlin *et al.*, 2010). The V1/V2 regions affect the sensitivity to neutralizing antibodies and evolve under positive natural selection *in vivo* (Sagar *et al.*, 2006). Studies have found that V1/V2 regions elongates during chronic infection as well as in Long-Term Non-Progressors (LTNPs), but not within rapid AIDS progressors (Shioda *et al.*, 1997; Kitrinis *et al.*, 2003; Curlin *et al.*, 2010).

Our laboratory has developed an approach to prepare a peptide construct that represents epitope variability in a single synthesis. HIV Hypervariable Epitope Constructs (HECs) are synthetic peptide mixtures that contain multiple variants of a given epitope, based on five variable regions of the gp120 envelope glycoprotein of HIV-1. In previous studies, we assessed the immunogenicity of HIV HECs in rhesus macaques and rabbits (Carlos *et al.*, 2000) and showed that HIV HECs are strong immunogens, eliciting both humoral and cellular immune responses. Our previous work also demonstrated that HIV-1 infected individuals with subtypes A, B, C, D, E and F from many different countries recognize all individual HIV HECs. Moreover, more than 96% of sera from a cohort (N = 1194) of HIV-1 positive and AIDS patients from Puerto Rico and Canada showed antibody reactivity to the HIV HECs (Carlos *et al.*, 1999).

In the present study, we wanted to determine to what extent LTNPs have antibodies that recognize the HIV gp120 hypervariable regions and if their antibody response would change during the time period examined.

Material and Methods

HIV HEC Design and Synthesis

Protein sequences corresponding to the five hypervariable immunogenic regions of HIV-1 gp120 were obtained from the Los Alamos National Laboratory for Human Retroviruses and AIDS, <http://www.hiv.lanl.gov/content/immunology>. The possible amino acids along the variable epitopes were determined from sequence information of viral isolates. In addition, HLA alleles that could bind to an epitope within HIV HECs were predicted based on HLA Ligand and Motif Database funded by NIH (Sathiamurthy *et al.*, 2003).

HIV HEC peptides representing the five hypervariable regions of gp120 were synthesized by 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry, a solid-phase peptide synthesis method, utilizing high capacity (0.7mmol/g) Knorr resin (Advanced Chemtech) as described previously (Carlos *et al.*, 2000; Kang *et al.*, 2010). Some amino acid coupling steps in the synthesis of the epitope were performed with a mixture of two amino acids as determined from sequence data. As a result, in a single synthesis, a HEC consisting of a mixture of peptides representing the major observed and

predicted *in vivo* variants of the epitope was produced. Each HEC contains 4, 6 or 32 variants of antigenic epitopes of hypervariable regions of gp120. Details of the preparation of HECs and analogs were described in a previous report (Carlos *et al.*, 2000). Single sequence peptides (analogs), representing the sequences found on HIV-1 envelope glycoprotein (gp120) of MN, RF and SF2 strains were also synthesized.

Plasma Collection from Long-Term Non-Progressors

Approximately 40-50 ml of blood (heparin coated tubes) was obtained by venipuncture from six LTNP individuals every two months over a course of 10 months. Three patient samples were collected at the Dominican Republic (two females and one male) and three in Puerto Rico (one female and two males). All six individuals have had documented HIV infection for 8-14 years and were asymptomatic with no opportunistic infections. Samples were collected under a protocol approved by the corresponding Institutional Review Board (IRB) in the Dominican Republic and Puerto Rico. All subjects gave written informed consent. These individuals were not receiving antiretroviral therapy.

Enzyme-Linked Immunosorbent Assay (ELISA)

The ELISA procedure was previously described (Kang *et al.*, 2010). All of the plasma samples from HIV LTNPs were heat-inactivated at 56°C for 30 min before testing. Antigens used were the five HIV HECs, a set of HEC analogs representing the sequences found on HIV-1 envelope glycoprotein (gp120) of MN, RF and SF2 strains, or recombinant protein gp120 (RF strain). Flat-bottomed microtiter plates (Corning Costar) were coated with HIV HECs and analog peptides at a concentration of 5 µg/well in triplicate at 37°C overnight; Recombinant gp120 protein was plated at a concentration of 1 µg/well. 10% non-fat milk was added to block non-specific binding for 2 h at 37°C and LTNP plasma was added at a 1:100 dilution in a volume of 50 µl per well. HIV-uninfected normal human serum was used as negative control. Antigen-bound antibodies were detected using anti-human IgG conjugated to Horseradish Peroxidase (HRP) at a 1:2000 dilution. SureBlue TMB solution was used as substrate (KPL). The O.D. was measured spectrophotometrically at 650nm with an automatic plate reader (VERS Amax, Molecular Devices) according to the manufacturer's instructions (SOFTmax PRO, Molecular Devices).

Statistical Analysis

The significance of difference in antibody responses between LTNPs and normal human serum was determined using unpaired Student's t-test. Data were regarded as statistically significant at the two-tailed p values of < 0.05.

Result

Plasma samples from three LTNP patients were obtained in Puerto Rico (PR) and another three samples in the Dominican Republic (DR).

To determine the extent and breadth of antibody binding to the HIV HECs, we performed ELISA using plasma from LTNPs. We also tried to determine whether the antibody response to HIV gp120 hypervariable regions changes over a 10-month period by examining two cohorts of three LTNPs (asymptomatic to AIDS and without opportunistic infections) from two different countries (Puerto Rico and the Dominican Republic).

All plasma samples from the six LTNPs showed positive antibody response to all of the individual HIV gp hypervariable regions over the course of the study, except for month 4 or 8 in patients PR1, PR3 and DR3 (Fig. 1). HIV negative, Normal Human Sera (NHS) were obtained from commercial sources or from HIV uninfected healthy individuals and used as a negative control. All six LTNP patients showed the strongest antibody response to HIV HEC 3 among all HIV HECs examined ($p < 0.001$) and the response was as high as or even greater than that obtained against recombinant gp 120 ($p < 0.001$). Moreover, the antibody response to HIV HEC 3 remained high during the course of study, showing no significant changes (Fig. 1). The antibody response to HIV HEC 1 was the second highest overall. Interestingly, one of the LTNP patients examined (DR1)

showed broadly reactive, strong antibody response to all of the individual HIV HECs and this patient maintained very low viremia over 4 years of follow-up (Fig. 1).

During the same period, we tested antibody responses to monovalent peptides (analogs) representing the same regions of gp120 as the HECs. The results one another, the antibody binding to HIV HECs was greater than to the analogs, including the sequences found in HIV-1 RF, SF2 and MN (Fig. 3). Similar results were observed for patient DR2, although the antibody response to the HIV HECs was not significantly higher than that to analogs except in months 0 and 2 (data not shown).

Patient DR3, with the highest viremia and the lowest ratio of CD4 to CD8 among all the patients examined, showed that regardless of the HIV-1 strain, the average antibody responses of LTNPs to analog epitopes of the V3 region of gp120 were the highest, compared to those to other analog epitopes (Fig. 4-6). Interestingly, this antibody response was weaker than to HIV HEC 3 ($p < 0.001$) (Fig. 2). In addition, when the antibody responses of patient DR1 to different antigens were compared to one another, the antibody binding to HIV HECs was greater than to the analogs, including the sequences found in HIV-1 RF, SF2 and MN (Fig. 3). Similar results were observed for patient DR2, although the antibody response to the HIV HECs was not significantly higher than that to analogs except in months 0 and 2 (data not shown).

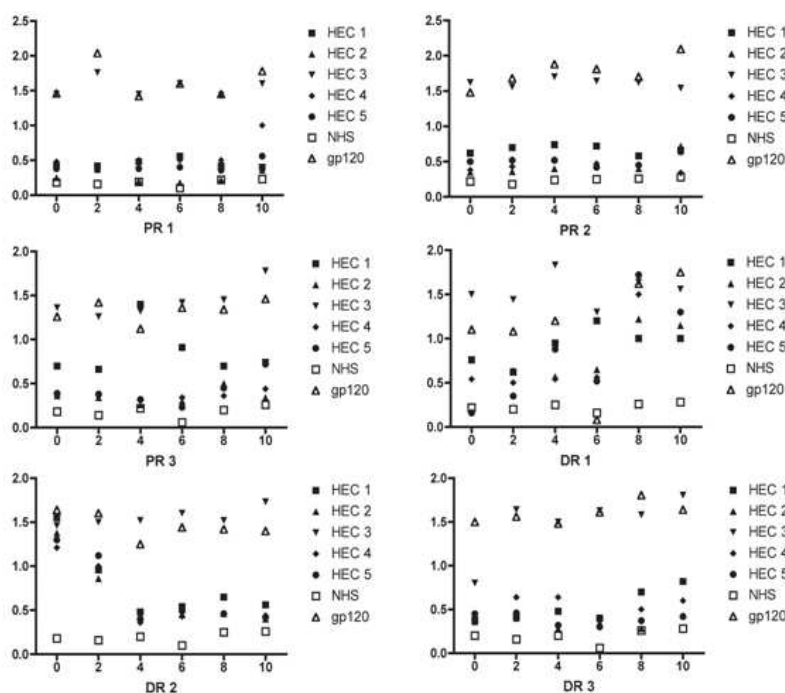


Fig. 1. Immune response of LTNPs to all HIV HECs at 0, 2, 4, 6, 8 and 10 months. All plasma samples from the six LTNPs showed positive antibody response to all of the individual HIV HECs over the course of the study, except for month 4 or 8 in patients PR1, PR3 and DR3. All six LTNP patients showed the strongest antibody response to HIV HEC 3 and HIV HEC 1 was the second highest. NHS: HIV-uninfected normal human serum. Gp120: Positive control. PR1, PR2, PR3: Plasma samples from three LTNP

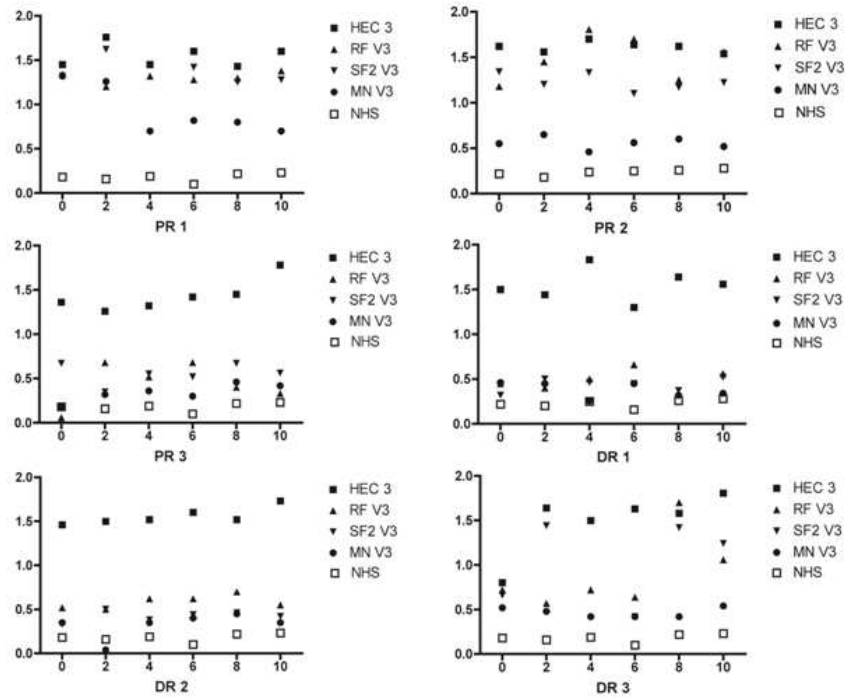


Fig. 2. Antibody responses to gp120 V3 region in 0, 2, 4, 6, 8, 10 months. All plasma samples from the six LTNP patients showed positive antibody response to Gp120 V3 region, but weaker than to HIV HEC 3. MN V3, RF V3, SF2 V3: Single sequence peptides (analogs), representing the V3 sequences found on HIV-1 envelope glycoprotein (gp120) of MN, RF and SF2 strains. NHS: HIV-uninfected normal human serum, as negative control. PR1, PR2, PR3: Plasma samples from three LTNP patients from Puerto Rico; DR1, DR2, DR3: Plasma samples from three LTNP patients from Dominican Republic. Values in the Y axis represent optical density

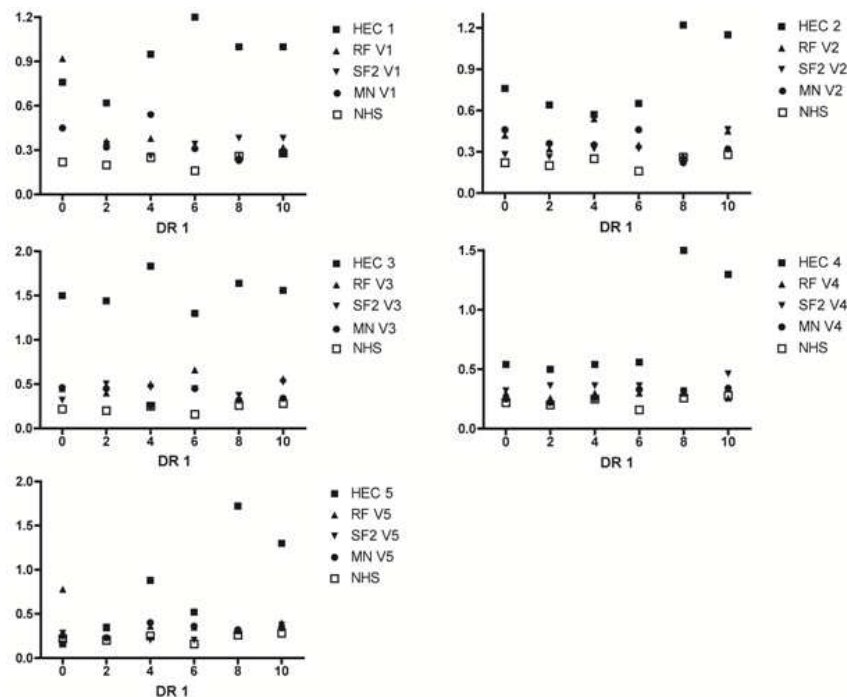


Fig. 3. Antibody responses of patient DR1 in 0, 2, 4, 6, 8, 10 months. Plasma sample from patient DR1 showed different level of antibody responses to the analogs of gp120 V1 to V5 regions, the antibody binding to HIV HECs was greater than to the analogs, including HIV-1 RF, SF2 and MN. Values in the Y axis represent optical density

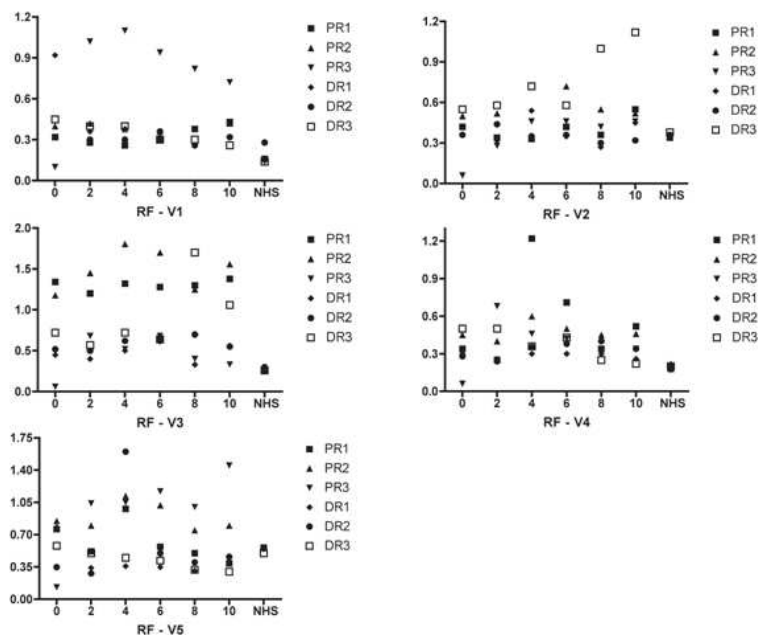


Fig. 4. Antibody responses to gp120 V1 to V5 of HIV1 RF strain in 0, 2, 4, 6, 8, 10 months. NHS: HIV-uninfected normal human serum. Values in the Y axis represent optical density

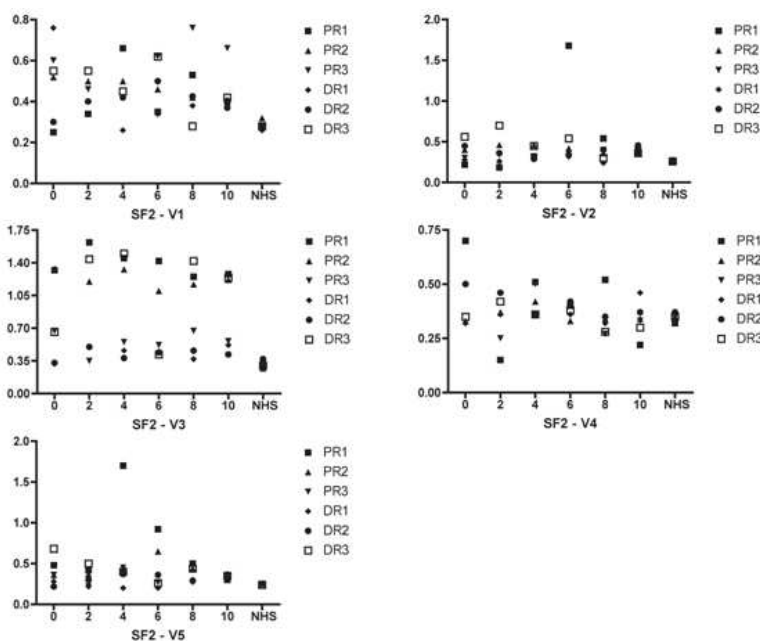


Fig. 5. Antibody responses to gp120 V1 to V5 of HIV1 SF2 strain in 0, 2, 4, 6, 8, 10 months. NHS: HIV-uninfected normal human serum. Values in the Y axis represent optical density

Patient DR3, with the highest viremia and the lowest ratio of CD4 to CD8 among all the patients examined, had a strong antibody response to the V2 epitope represented by all three analogs tested. PR3 showed a strong antibody response to the V1 epitope of analogs SF2 and RF, but not to MN, which are X4R5 dual tropic and X4 tropic HIV-1 viruses, respectively. Another interesting finding was that the antibody response to all

three analogs of the V3 epitope from patients PR3 and DR1 was relatively lower than the responses of patients PR1, PR2 and DR3 considering that PR3 and DR1 showed the lowest viremia among the patients examined. Patient PR1 showed a transient, strong antibody response to epitopes from V2, V4 and V5, but we could not find any correlation between these antibody responses and viremia or the ratio of CD4 to CD8 (Fig. 4-6).

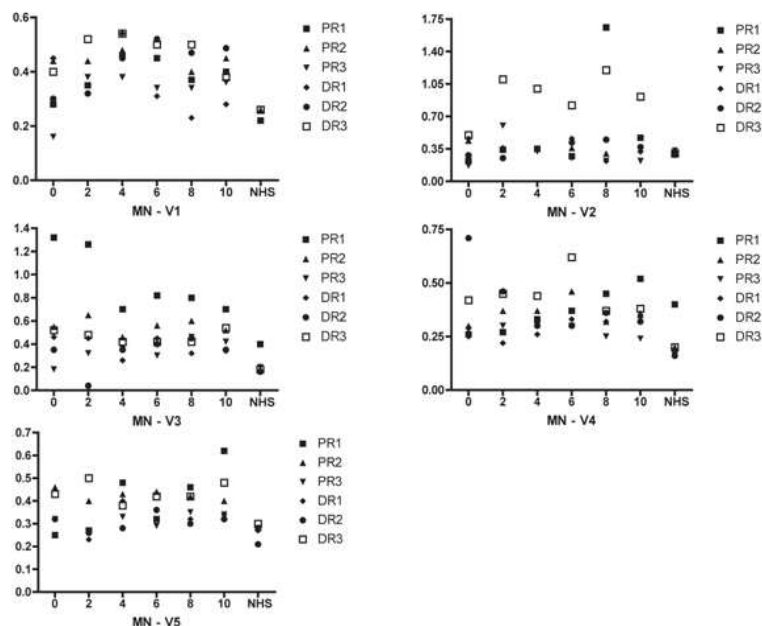


Fig. 6. Antibody responses to gp120 V1 to V5 of HIV1 RF strain at 0, 2, 4, 6, 8 and 10 months. NHS: HIV-uninfected normal human serum. Values in the Y axis represent optical density

Discussion

HECs was stronger than to the monovalent peptides that represent single strains of HIV-1 (analogs). It is apparent from the present longitudinal study of a few LTNP patients that these individuals mount a humoral immune response that includes antibodies specific to the five variable epitopes studied. Probably due in part to the different genetic HLA background of each patient, the recognition of HIV HECs and analog epitopes is highly variable and unique.

V3 sequence variability affects virus tropism by determining which co-receptor, CXCR4 or CCR5, is used by HIV to infect target cells (Jensen and van't Wout, 2003) (Jensen *et al.*, 2003). In addition, amino acid substitutions in the V3 loop are associated with the switch from Non-Syncytium-Inducing (NSI) variants to Syncytium-Inducing (SI) variants (Wang *et al.*, 2000; Edo-Matas *et al.*, 2011). A disulfide bond formed by two cysteine residues flanking the V3 sequence generates a loop conformation and the tip of the V3 loop consists of a conserved amino acid sequence (Su *et al.*, 2000; Huang *et al.*, 2005). Several studies have demonstrated that antibodies elicited against hypervariable and conserved epitopes in the V3 loop showed broadly-

reactive neutralization to multiple subtypes of HIV-1 (Zolla-Pazner, 2004; Hioe *et al.*, 2010; Swetnam *et al.*, 2010). In the present study, we also observed that antibody responses to HIV HEC 3 and HEC analog V3 were the strongest, compared to those to other HECs and analogs. However, we did not test to what extent and breadth the antibodies that reacted to HIV HEC V3 were involved in the induction of cross-reactive neutralization to variable or conserved epitopes.

For the patients studied here, we do not know the sequence of the infecting virus; thus, a correlation between the epitopes represented in the viral strain and the patients' response could not be assessed at this time. Moreover, the antibody responses from individual LTNP patients were extremely variable to different antigens, including HIV HECs and analog peptides, HIV-1 RF, SF2 and MN, during the time period examined. This extreme variability in the antibody responses of individual LTNP patients made it difficult to find any correlation between antibody responses and viremia or CD4:CD8 ratios. However, it is very likely that each person was infected by a different strain or a different collection of strains. This may contribute to the stronger antibody response to HIV HECs than to analogs representing single strains of HIV-1. As samples from truly untreated LTNPs are very hard to obtain, these studies only provide a small window into the immunological, virological and clinical parameters documented over time in these unique patients. Further studies with a larger number of patient samples is needed to determine whether patients with low viremia have strong antibody responses to the V2 epitope of

gp120, or have stronger antibody responses to HIV HECs (variable epitopes) than patients with high viremia. Interestingly, two genetic signatures at two amino acid positions in V2 were found to be less common in individuals infected after vaccination with the RV144 vaccine (Rolland *et al.*, 2012). Additional information concerning T helper, cytotoxic and suppressor responses needs to be obtained to further understand the correlation between the immune responses to HIV gp120 hypervariable epitopes and the long delay to progression to AIDS observed in LTNPs.

HIV HECs represent highly variable antigenic epitopes from all five variable regions of the HIV-1 envelope glycoprotein 120. Barouch *et al.* (2010) demonstrated that a bivalent mixture of mosaic HIV-1 proteins consisting of two HIV-1 Gag, Pol and Env antigens elicited stronger cellular immune responses to variant T cell epitope peptides in rhesus macaques than those of naturally occurring or consensus antigens (Barouch *et al.*, 2010). This experimental design is similar to that employed in our previous studies (Carlos *et al.*, 1999; 2000; Azizi *et al.*, 2008; Kang *et al.*, 2010), though Barouch *et al.* (2010) used antigenic epitopes containing more limited variability in sequence polymorphism.

The fact that antibodies from some LTNPs reacted more strongly to individual HIV HECs than to single strain peptides proves that HIV HECs enhance the sensitivity of recognition of antibodies elicited by HIV-1 infection. This information could contribute to the development of an immunogen to be included in the design of preventive and therapeutic vaccine approaches against HIV-1 infection and AIDS.

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Author's Contributions

Rebecca Rivera and Kyung Hee Kang: Performed experiments and data interpretation. Participated in manuscript preparation.

Murray B. Gardner and David E. Anderson: Performed data interpretation. Participated in manuscript preparation.

Santiago Collado-Chastel, Eddy Rios-Olivares and Yasuhiro Yamamura: Collected, categorized and provided clinical samples.

Francisco Diaz-Mitoma: Performed data interpretation.

Xia Li: Performed data interpretation. Participated in manuscript revision.

José V. Torres: Planned the project and directed the research group.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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