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Low frequency of broadly neutralizing HIV antibodies during chronic infection even in quaternary epitope targeting antibodies containing large numbers of somatic mutations

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ABSTRACT

Neutralizing antibodies (Abs) are thought to be a critical component of an appropriate HIV vaccine response. It has been proposed that Abs recognizing conformationally dependent quaternary epitopes on the HIV envelope (Env) trimer may be necessary to neutralize diverse HIV strains. A number of recently described broadly neutralizing monoclonal Abs (mAbs) recognize complex and quaternary epitopes. Generally, many such Abs exhibit extensive numbers of somatic mutations and unique structural characteristics. We sought to characterize the native antibody (Ab) response against circulating HIV focusing on such conformational responses, without a prior selection based on neutralization. Using a capture system based on VLPs incorporating cleaved envelope protein, we identified a selection of B cells that produce quaternary epitope targeting Abs (QtAbs). Similar to a number of broadly neutralizing Abs, the Ab genes encoding these QtAbs showed extensive numbers of somatic mutations. However, when expressed as recombinant molecules, these Abs failed to neutralize virus or mediate ADCVI activity. Molecular analysis showed unusually high numbers of mutations in the Ab heavy chain framework 3 region of the variable genes. The analysis suggests that large numbers of somatic mutations occur in Ab genes encoding HIV Abs in chronically infected individuals in a non-directed, stochastic, manner.

1. Introduction

An increasing number of broadly neutralizing monoclonal Abs against HIV have been identified in recent years that recognize novel neutralizing epitopes on the envelope (Env) glycoprotein complex. However, broadly neutralizing Ab clones are relatively rare and occur late in infection. Generally, it is thought that the broadly neutralizing epitopes may be relatively poorly immunogenic, difficult to access, and they require extensive somatic hypermutation for targeting (Burton et al., 2005). It has been postulated that conformationally dependent Abs against intact native trimers may be necessary to neutralize HIV. This proposal is consistent with studies that show much of the neutralizing activity to HIV is directed to quaternary epitopes (Steimer and Haigwood, 1991; Steimer et al., 1991). Recent studies show certain constructs of recombinant proteins that seem to have improved specificity for representing neutralizing epitopes (Sanders et al., 2013).

Conformationally or structurally dependent epitopes can take many forms, from discontinuous amino acids in a short sequence, to widely separated amino acids brought together by protein folding, or epitopes present only during the arrangement of multiple protomers, *i.e.* quaternary epitopes. The quaternary epitopes may

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be formed by portions of a single protein of the complex or quaternary epitopes may be formed *de novo* between the units of the multiple protomers. Structural antigenic determinants that induce quaternary-targeted Abs, have been shown to be important in other infections. For example, less than 1% of dengue virus-specific human mAbs are serotype-specific and potent neutralizing Abs, and these recognize quaternary structures that are found only on virion particles, not on soluble E protein (Fibriansah et al., 2014; Messer et al., 2014; de Alwis et al., 2012; Fibriansah et al., 2015). The potent and broad influenza-specific human mAb 1F1 recognizes a quaternary epitope on the hemagglutinin protein head domain (Tsibane et al., 2012). Large-scale efforts to identify potent and broadly neutralizing anti-HIV Abs against HIV using neutralization assays for the screen instead of Ab binding assays have recently isolated the broadly neutralizing Abs PG9 and PG16 (Walker et al., 2009) that target the same epitope as the quaternary targeting antibody (QtAb) 2909 (Gorny, 2005). PG9, PG16 and 2909 all target a variable loop 1 and 2 dependent epitope present on gp120 monomers (McLellan et al., 2011), although the binding is greatly enhanced by incorporation into the envelope trimeric spike and have been referred to as quaternary-structure-preferring Abs (McLellan et al., 2011; Davenport et al., 2011). Other HIV Abs that recognize complex structures have been identified recently (Pietzsch et al., 2010; Louis et al., 2014; Gustchina et al., 2013; Scharf et al., 2014), including the broadly neutralizing QtAb PGT-151 (Blattner et al., 2014). PGT-151 binds an epitope that is only created between gp120 and gp41 protomers after cleavage and trimeric incorporation. As a class, however, broadly neutralizing Abs, particularly those that target complex quaternary epitopes, seem to be rare occurrences.

HIV produces a membrane-enveloped virion studded with HIV Env protein. We previously developed a pseudovirion- or virus-like particle (VLP)-based platform for antigen presentation of naturally cleaved Env trimer protein (Hammonds et al., 2007). We subsequently demonstrated that the VLPs presented the epitopes for classical broadly neutralizing HIV mAbs and responded to CD4 binding by increasing access to CD4-induced epitopes (Hicar et al., 2010a). We previously used these VLPs in flow cytometric cell sorting experiments to isolate a neutralizing human mAb to a CD4-induced quaternary epitope (Hicar et al., 2010b). Virus-like particles (VLPs) have been considered as vaccine candidates, however these constructs are not without issue. Production of VLPs can incorporate other host membrane proteins that also induce immune responses (Hammonds et al., 2007). Nonfunctional gp120/gp41 monomers and gp120-depleted gp41 stumps also may be displayed on VLPs as they are on virions (Crooks et al., 2007). These altered proteins have been proposed to be a component of diversionary tactics to elicit an inefficient Ab response against the virus.

It is poorly understood whether a subset of quaternary epitope-targeting Abs (QtAbs) are neutralizing or whether members of this class of Abs are usually neutralizing. We sought here to address that question by isolating panels of human mAbs that target quaternary epitopes on trimeric HIV Env, without prior screening for neutralization, using our VLPs in flow cytometric B cell capture assays. We identified novel anti-HIV Abs from three different HIV-infected long-term non-progressor subjects. On initial screens, a surprising number of these Abs targeted complex structural epitopes, *i.e.*, quaternary epitopes. As a class, these QtAbs exhibited a very high level of somatic hypermutation in the Ab variable genes. Despite the fact that the majority of Abs isolated by selection with VLPs were QtAbs, none of these Abs exhibited broad or potent HIV neutralizing or Ab-dependent cell-mediated viral inhibition (ADCVI) activity when expressed as full-length immunoglobulins. These QtAbs did display interesting common molecular characteristics, such as the dominant use of particular Ab variable gene segments and extensive mutations away from the inferred germline genes, particularly in the framework 3 region. We sought to examine the idea that extensive somatic mutations and induction of QtAbs would be sufficient to identify broadly neutralizing Abs. This was not the case, however. Mutational and biochemical analysis revealed these framework 3 region mutations accumulated stochastically in a manner that did not contribute to enhanced virus inhibiting activity. The results suggest that although expansion of B cells encoding QtAbs with large numbers of somatic mutations is often necessary for development of neutralizing Abs, these features are not sufficient to confer such activity. Rather, only rare clones of this class of Ab are likely to mediate broad and potent neutralizing activity due to particular patterns of epitope recognition.

2. Materials and methods

2.1. Human subjects

Whole blood was obtained from subjects having clinical care at the Comprehensive Care Center at Vanderbilt University Medical Center, Nashville, TN after informed consent. Samples were de-identified as to age, gender, and other personal identifying information. The Institutional Review Board of Vanderbilt University Medical Center approved the study protocols and consent forms. Subjects 10002, 10028, and 10076 were infected chronically with HIV, with a duration of infection from 7 to 24 years, and with CD4+ T cell counts >500 at the time of B cell sorting. These subjects were treatment-naïve, as noted in previous publications (Sather and Stamatatos, 2010).

2.2. Generation of green-fluorescent protein-labeled virus—like particles (GFP-VLP) for single-cell sorting of HIV-specific CD19 ± B cells

Stable cell lines were generated as previously described (Hicar et al., 2010b). Briefly, plasmid pcDNA4/TO containing sequence-optimized HIV-1 *gag*, IRES and HIV-1 BaL *env* genes was created in the T-Rex-293 cell line (Invitrogen, Carlsbad CA). The GFP gene was cloned in-frame with the HIV-1 *vpr* gene and cloned into the pcDNA5/TO vector; transfected into the pcDNA4/TO Gag-I-Env stable cell line; and, using blasticidin, hygromyin, and zeocin, selected for stable cell line production. Cell lines were selected for optimal coordinated gag and env production after doxycycline induction and the optimal cell line was designated XC-34.

GFP-labeled VLPs were harvested from individual clones after three days of 2 μg/mL doxycycline induction and clarified by low-speed centrifugation, filtered through a 0.45 μm filter, and then purified by ultra centrifugation through a 20% sucrose cushion (100000 × *g* for 2 h, 4 °C). Pellets were suspended in PBS. The gp120 and p24 antigen content were measured by antigen-capture ELISA techniques.

2.3. B cell labeling, single-cell sorting of HIV VLP-binding CD19± B cells

B cell sorting was performed as previously described (Hicar et al., 2010b). Peripheral blood lymphocytes were isolated by centrifugation on 1.078 density lymphocyte separation medium. $CD19^+$ B cells were separated using paramagnetic beads according to the manufacturer's instructions (STEMCELL Technologies, Vancouver, BC). Then, 2 to 4×10^6 B cells were stained with anti-CD3-PE, anti-CD14-PE, anti-CD19-APC (Becton Dickinson, Franklin Lakes, NJ) and 50 μL of concentrated VLP (containing GFP) preparation on ice for 30 min. Flow cytometric analysis and single-cell sorting was performed with a FACSAria III flow cytometer in a Biosafety Level 3 laboratory, equipped with an automated single-cell deposition unit and aerosol containment accessory (Becton Dickinson,