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Dynamic evolution of antibody populations in a rhesus macaque infected with attenuated simian immunodeficiency virus identified by surface plasmon resonance

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Abstract

Background—Increasing evidence suggests that an effective AIDS vaccine will need to elicit broadly neutralizing antibody responses. However, the mechanisms of antibody-mediated neutralization have not been defined. Previous studies from our lab have identified significant differences in the rates of antibody binding to trimeric SIV envelope proteins that correlate with neutralization sensitivity. Importantly, these results demonstrate differences in monoclonal antibody (MAb) binding to neutralization-sensitive and neutralization-resistant envelope proteins, suggesting that one mechanism for virus neutralization may be related to the stability of antibody binding. To date, little has been done to evaluate the binding properties of polyclonal serum antibodies elicited by SIV infection or vaccination.

Methods—In the current study, we translate these findings with MAbs to study antibody binding properties of polyclonal serum antibody responses generated in rhesus macaques infected with attenuated SIV. Quantitative and qualitative binding properties of well-characterized longitudinal serum samples to trimeric, recombinant SIV gp140 envelope proteins were analyzed using surface plasmon resonance (SPR) technology (Biacore).

Results—Results from these studies identified two antibody populations in most of the samples analyzed; one antibody population exhibited fast association/dissociation rates (unstable) while the other population demonstrated slower association/dissociation rates (stable). Over time, the percentage of the total binding response of each antibody population evolved, demonstrating a dynamic evolution of the antibody response that was consistent with the maturation of antibody responses defined using our standard panel of serological assays. However, the current studies provided a higher resolution analysis of polyclonal antibody binding properties, particularly with respect to the early time-points post-infection (PI), that is not possible with standard serological assays. More importantly, the increased stability of the antibody population with time PI corresponded with potent neutralization of homologous SIV *in vitro*.

Conclusions—These results suggest that the stability of the antibody–envelope interaction may be an important mechanism of serum antibody virus neutralization. In addition, measurements of

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the ‘apparent’ rates of association and dissociation may offer unique numerical descriptors to characterize the level of antibody maturation achieved by candidate vaccine strategies capable of eliciting broadly neutralizing antibody responses.

Keywords

antibody; Biacore; binding; envelope; SIV

Introduction

A protective AIDS vaccine will require both enduring and broadly protective cellular and humoral immune responses to maximize the potential for protection from many HIV-1 variants by different routes of exposure. Increasing evidence for the role of antibody responses in protective immunity comes from passive serum/antibody experiments where the protective efficacy of antibodies against viral exposure in the HIV-1/chimp [14, 38], SIV/monkey [8, 22, 23, 47, 49], and simian-human immunodeficiency virus (SHIV)/monkey [3, 20, 28, 29, 32, 33, 45] systems has been demonstrated. In addition, neutralizing human monoclonal antibodies (MAbs) directed to conformational epitopes in the HIV-1 envelope conferred protection against both mucosal and intravenous routes of exposure to SHIV [3, 32]. Further evidence for cross reactive neutralizing antibody responses in long-term non-progressors suggests that antibody may also be involved in the control of virus infection and prevention from disease progression [5]. These studies demonstrate the ability of neutralizing antibodies alone to mediate protection against pathogenic challenge, and suggest a role for neutralizing antibody responses during the chronic stage of infection.

Immune correlates of vaccine protection have been typically based on standardized *in vitro* assays of antibody titer to a particular antigen, serum neutralizing activity in a defined virus/target cell system, and/or the level of cellular immune responses to a defined vaccine immunogen. Not only do these classical assays fail to define reliable immune correlates of protection, cumulative data suggest that these *in vitro* assays are not measuring what is relevant to the presence or absence of immune protection *in vivo* [4]. The past decade has seen the development of several new assays, including tetramer staining [1, 21, 27], flow cytometry [16, 34, 35] and enzyme-linked immunosorbent spot-forming cell assay [37, 44] that have improved the specificity and sensitivity of cellular immune responses over the conventional cytotoxic T lymphocyte assays. To address the need for additional assays to evaluate virus-specific antibody responses, we have during the past several years developed two new assays to measure the qualitative properties that complement the existing quantitative assays of antibody titers. Using these new antibody assays in the SIV/macaque model, we defined a novel maturation of antibody responses characterized by ongoing changes in antibody avidity and conformational dependence that continued long after maximum titers had been achieved [8, 11]. This maturation process was also associated with the development of protective immunity in monkeys infected with attenuated SIV [8]. Interestingly, while neutralizing antibody titers to the homologous virus emerge rapidly after infection, it is not until this antibody maturation is achieved that emergence of neutralizing antibody responses to the heterologous challenge virus is evident [8]. Furthermore, studies in the HIV-1 [10], SHIV [10] and equine infectious anemia virus (EIAV) [24] systems suggest that this antibody maturation process is a common property of lentiviruses [36].

These serological antibody assays have provided important information about the maturation of envelope-specific antibody responses to SIV, SHIV and HIV-1 envelope proteins as well as correlated with the development of protective immunity in the SIV system. However, we recognize the limitations of these solidphase assays, including the difficulty in obtaining reproducible data when only minor changes to the assay are introduced. For this reason, we

have recently developed antibody binding assays based on surface plasmon resonance (SPR) that can provide increased sensitivity and reproducibility. Using SPR, we have recently identified that the kinetic rates of MAb binding correlated with neutralization of SIV [46]. In the current study, we translate these findings with MAbs to a longitudinal panel of polyclonal serum from a rhesus macaque infected with attenuated SIV. Results from these studies demonstrate for the first time a more discriminating evaluation of polyclonal antibody responses using SPR compared with standard serological assays to define maturation, particularly in the early time-points PI, which may correlate with an increased breadth in neutralization. This finer resolution analysis of the qualitative antibody binding properties in 'real time', independent of the quantity of antibody, maximized our ability to characterize properties of antibody even in very early, low titer samples. Further, these studies suggest that SPR analyses may provide numerical descriptors (i.e., apparent dissociation rates) that can be used to characterize the level of antibody maturation elicited by experimental infection or vaccination.

Materials and Methods

Animals

Four rhesus macaques were inoculated intravenously via the saphenous vein with 10 TCID₅₀ of a cryopreserved preparation of cell-free SIV/17E-Fred [19] propagated in rhesus lymphocytes. All monkeys seroconverted and became persistently polymerase chain reaction (PCR) positive following infection. Infection was determined by PCR amplification of viral sequences. Animals were cared for in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals, the Animal Welfare Act, and protocols approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Viral load determination

Quantitation of virion-associated RNA in plasma was performed by real-time PCR in a Prism 7700 sequence detection system (Applied Biosystems, Inc., Foster City, CA, USA). Virions were pelleted from 1 ml of plasma by centrifugation at 14,000 *g* for 1 hour. Total RNA was extracted from the virus pellet by using Trizol reagent (Life Technologies, Rockville, MD, USA), and 20 μ l of each sample was analyzed in a 96-well plate. Synthesis of cDNA was accomplished in triplicate reactions in a mixture containing 50 mM MgCl₂, 1x PCR buffer II (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 0.75 mM dGTP, 0.75 mM dATP, 0.75 mM dCTP, 0.75 mM dTTP, 1 U of RNase inhibitor, 1.2 U of murine leukemia virus reverse transcriptase (RT), 2.5 μ M random hexamers, and 10% total viral RNA. Samples were mixed and incubated at room temperature for 10 minutes followed by 42°C for 12 min. The reaction was terminated by heating at 99°C for 5 minutes and incubating at 4°C for 5 min. The PCR was initiated immediately after the addition of RT by adding 30 μ l of a PCR master mix containing 1x PCR buffer A, 5.5 mM MgCl₂, 2.5 U of Amplitaq Gold, 200 mM deoxyribonucleoside triphosphates (dNTPs), 450 nM each primer, and 200 nM probe. The primers used were: 5'-AGGCTGGCAGATTGAGCCCTGGGAGGTTTC- 3' and 5'-CCAGGCGGCGACTAGGAGAGATGGGAACAC- 3', and the probe used was 5'-TTCCCTGCTAGACTCTCACCAGCACTTGG- 3'. The probe was labeled in the 5' position with the fluorescent reporter dye 6-carboxyfluorescein and in the 3' position with the quencher dye 6-carboxymethylrhodamine.

The amplification was carried out in the Prism 7700 sequence detection system by heating the specimens at 95°C for 10 minutes to activate Amplitaq Gold (Perkin- Elmer, Foster City, CA, USA), prior to subjecting them to 40 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 30 s. Serial dilutions of RNA obtained by *in vitro* transcription of a long terminal repeat

(LTR)- containing plasmid, ranging from 10^8 to 10^0 copies/reaction, were subjected to reverse transcription-PCR in triplicate, along with the samples, to generate a standard curve with a sensitivity threshold of 10 copies/reaction. RNA copy numbers from the unknown plasma samples were calculated from the standard curve and expressed as RNA copies per milliliter of plasma.

DNA sequencing of SIV V1/V2

The V1/V2 sequence was amplified in duplicate reactions by nested PCR from proviral DNA extracted from serially sampled peripheral blood mononuclear cells (PBMC). PCR products from positive duplicate reactions were pooled and cloned into the TOPO TA vector as per manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Eight to 11 colonies were randomly selected and sequenced in an ABI 370 automated DNA sequencer (Applied Biosystems). Sequences were prepared for publication using SeqPublish (Los Alamos National Laboratory, <http://hiv-web.lanl.gov/content/index>).

Virus production

SIVmac239 and SIV/17E-CL virus stocks were prepared by transfection of CEMx174 cells in 75 cm² culture flasks (BD Falcon, Bedford, MA, USA). Supernatant fluids were collected 1 week after transfection, filtered, aliquoted, and stored at -80°C . The infectious titer of each virus stock was determined by incubating 10-fold dilutions of culture supernatants containing SIVmac239 or SIV/17E-CL with TZM-bl cells for 48 hours at 37°C . TZM-bl cells are a HeLa-derived cell line engineered to express human CD4, CCR5, and CXCR4 as well luciferase expression that is driven by the HIV-1 long terminal repeat upon infection of the cells with HIV or SIV strains [51]. Following infection, supernatants were removed and the cells were lysed with 50 μl lysis buffer (25 mM Tris-phosphate, pH 7.8; 2 mM DTT; 2 mM 1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid; 10% glycerol; 1% Triton X-100) for 15 minutes at room temperature. A volume of 40 μl of the cell lysates were transferred to a white luminescence plate (USA Scientific, Ocala, FL, USA), 20 μl luciferase substrate (Promega, Madison, WI, USA) was added to each well, and the samples were assayed for luciferase production using a Lmax II luminometer (Molecular Devices, Sunnyvale, CA, USA). The 50% tissue culture infectious dose for each virus stock was determined using the method of Reed and Muench [40].

ConA ELISA

We determined the reactivity of serum samples from infected rhesus macaques to SIV envelope proteins in a ConA enzyme-linked immunosorbent assay (ELISA) as previously described [11]. Briefly, Triton-disrupted SIVsmB7 [8, 26] virions were captured for 1 hour at room temperature onto Immulon 2HB microtiter plates (Thermo Electron Corp., Waltham, MA, USA) coated with Concanavalin A (ConA) (Sigma, St Louis, MO, USA). Following ConA capture, all wells were washed with phosphate-buffered saline (PBS) and blocked by the addition of 5% dried milk in PBS (blotto) for 1 hour at room temperature. Rhesus serum samples were serially diluted twofold in blotto and incubated in the SIV envelope-coated wells for 1 hour at room temperature. Following extensive washing, peroxidase-conjugated anti-monkey IgG (Nordic Immunology Laboratories, Tilburg, The Netherlands) was diluted in blotto, added to each well and incubated for 1 hour at room temperature. After a final washing step, all wells were incubated with TM Blue substrate (Serologicals Corp., Norcross, GA, USA) for 20 minutes at room temperature, color was developed by the addition of 1 N sulfuric acid, and the wells were read at an optical density (OD) of 450 nm using an automated ELISA plate reader (Thermo Electron Corp.). Endpoint titers were determined as the last twofold dilution where the OD was twice that of normal monkey serum at the lowest dilution (1:50) or an OD of 0.100, whichever was greater. Conformational dependence was determined by measuring the reactivity to native vs.

denatured envelope proteins, where a ratio of >1 reflects predominant reactivity with native envelope proteins and a ratio of <1 reflects predominant reactivity with denature envelope proteins. Antibody avidity was determined by measuring the stability of the antigen-antibody complexes to 8 M urea and is expressed as follows: percent antibody avidity = $(\text{OD of wells washed with 8 M urea} / \text{OD of wells washed with PBS}) \times 100$. The results represent the average of at least three independent experiments, with variation in individual antibody avidity and conformational dependence values of $<10\%$.

Virus neutralization determination

TZM-bl cells [50] were plated at 10^4 cells per well in a cell culture-treated 96-well plate (BD Falcon, Bedford, MA, USA) and incubated for 6–8 hours at 37°C prior to infection. MAb dilutions were prepared in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS), with a starting concentration of $20\text{ }\mu\text{g/ml}$ and serially diluted fivefold to a concentration of $0.00128\text{ }\mu\text{g/ml}$ in a sterile 96-well V-bottom plate (Sarstedt, Newton, NC, USA). For MAbs that exhibited minimal or no neutralization, the assay was repeated using a starting concentration of $50\text{ }\mu\text{g/ml}$ and serially diluted fivefold to a concentration of $0.0032\text{ }\mu\text{g/ml}$. Virus was diluted in DMEM with 10% FBS to yield a multiplicity of infection of 0.01. A volume of $100\text{ }\mu\text{l}$ diluted virus was added to the MAb dilutions, yielding final concentrations of MAbs of $10\text{--}0.00064\text{ }\mu\text{g/ml}$ ($25\text{--}0.0016\text{ }\mu\text{g/ml}$), and incubated at 37°C for 1 hour. After 1 hour, media was removed from the TZM-bl cells and $200\text{ }\mu\text{l}$ of the virus-MAb mixture was added to the cells. Cells were incubated with the virus-MAb mixture for 48 hours at 37°C . After 48 hours, the virus-MAb mixture was removed and cells were lysed in $50\text{ }\mu\text{l}$ lysis buffer (25 mM Tris-phosphate, pH 7.8; 2 mM DTT; 2 mM 1,2-diaminocyclohexane- *N,N,N',N'*-tetraacetic acid; 10% glycerol; 1% Triton X-100) for 15 minutes at room temperature. A volume of $40\text{ }\mu\text{l}$ of the lysate was transferred to a white luminescence plate (USA Scientific) and the samples were assayed for luciferase production using a Lmax II luminometer (Molecular Devices), where $20\text{ }\mu\text{l}$ luciferase substrate was automatically injected into each sample immediately prior to reading. 50% neutralization titers were determined using method of Karber [25].

Recombinant envelope protein production and purification

SIVmac239 [41] and SIV/17E-CL [2] recombinant gp140 proteins were expressed and produced from vaccinia virus infection of 293T cells as previously described [6, 18]. Briefly, the envelope gene from SIV/17E-CL was cloned into pSC65 (kindly provided by Dr Bernard Moss, National Institutes of Health, Bethesda, MD, USA), and a premature stop codon just N-terminal to the membrane-spanning domain was introduced using PCR-based mutagenesis. Recombinant vaccinia viruses were generated from transfection of these plasmids into HeLa cells infected with the Western Reserve wild-type vaccinia virus strain using standard techniques [17]. The virus containing SIVmac239 envelope (vCB74) was produced using the same protocol and was kindly provided by Dr Robert Doms (University of Pennsylvania, Philadelphia, PA, USA) [18]. Recombinant vaccinia viruses were used to infect 293T cells at a multiplicity of infection of 10, the secreted envelope proteins harvested as cell culture supernatants and purified with lentil lectin chromatography using 0.5 M methyl α -D-mannopyranoside (Sigma) for elution as previously described [6].

CD4 binding ELISA

The reactivities of viral or recombinant gp140 SIVmac239 and SIV/17E-CL envelope antigens to recombinant soluble CD4 (sCD4) were determined using a ConA ELISA. Envelope proteins were captured onto ConA-coated plates as described above. All wells were blocked with 5% BSA before being incubated with 10 ng/well sCD4 (provided by the AIDS Research and Reference Reagent Program, NIH, Rockville, MD, USA) for 1 hour at 25°C . All wells were incubated with a 1:1000 dilution of a rabbit anti-CD4 antibody

(provided by AIDS Research and Reference Reagent Program) for 1 hour at 25°C, followed by incubation with peroxidase-labeled goat anti-rabbit IgG (Nordic Immunology Laboratories) for 1 hour at 25°C. TM Blue substrate (Serologicals Corporation, Gaithersburg, MD, USA) was added to each well for 20 minutes at room temperature, color development was stopped by the addition of 1 N sulfuric acid, and the OD at 450 nm was read using an automated ELISA plate reader (Thermo Electron Corp.).

Native-polyacrylamide gel electrophoresis and Western blot analysis

The recombinant envelope proteins were resolved on a Criterion XT Tris-acetate 3–8% continuous-gradient polyacrylamide gel (Bio-Rad, Hercules, CA, USA) before being transferred to polyvinylidene difluoride membranes. Recombinant viral envelope proteins were detected using an MAb specific for SIV envelope, rhesus MAb 3.11H [9]. Properly multimerized trimeric proteins were visualized as a band at approximately 420 kDa when compared with migration of High Molecular Weight Native Markers (Amersham Biosciences, Buckinghamshire, UK). gp120 was also run as a control to visualize monomeric protein in the event the trimeric proteins exhibited other non-trimeric forms.

Surface plasmon resonance analysis

To assess the binding properties of serum antibodies we utilized SPR technology using a Biacore 3000 (Biacore AB, Uppsala, Sweden). Protein A (Pierce, Rockford, IL, USA) was immobilized to the surface of a CM5 sensor chip (Biacore, Inc., Piscataway, NJ, USA) using standard amine coupling chemistry. The surface of the chip was activated using a 1:1 mixture of *N*-hydroxysuccinimide and 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC) (Biacore, Inc.) followed by a 20-minute injection of protein A (70 µg/ml) to capture ~5000 RU of protein A on two adjacent flowcells on a CM5 sensor chip. Remaining active carboxyl groups were inactivated with an injection of 1 M ethanolamine, and remaining non-covalently associated protein A was washed from the surface using four 30 s injections of 100 mM HCl (100 µl/min). The second flowcell of the protein A-coated CM5 sensor chip was then used to capture polyclonal IgG antibody from longitudinal serum samples (1, 2, 3, 4, 5, 8, and 12 months PI) from SIV/17E-CI-infected rhesus macaque #1998. Serum was diluted in HBS-EP buffer such that a 5 µl injection at a flow rate of 10 µl/min yielded ~100 RU IgG antibody captured by the protein A on the CM5 chip. 400, 200, and 200 RU antibody was captured for 1-, 2-, and 3-month PI samples, respectively, reflecting the lesser amount of SIV envelope-specific antibody present in the serum at those early time points compared with months 4–12 PI. After capture of IgG, varying concentrations (0.27–66.67 nM, series of threefold dilutions) of SIV/17E-CI trimeric gp140 were passed sequentially over both flowcells of the sensor chip. A blank (0 nM) injection was also included. Binding isotherms were then analyzed using BiaEvaluation 4.1 (Biacore AB). Due to the observation of two antibody populations using the avidity ELISA described above, all samples were initially fit to the Heterogeneous Ligand binding model. Where this model returned two populations with identical apparent binding kinetics, the binding curves were refit to the 1:1 Langmuir binding model. It is important to realize that kinetic rates returned using these binding models for polyclonal serum represent only apparent rates of binding due to the multiple specificities inherent to a polyclonal response and do not define the kinetics of the polyclonal antibody anti-SIV envelope response.

Results

Rhesus macaques infected with attenuated SIV/17E-Fr achieve a mature antibody response as defined by a panel of serological assays

Four rhesus macaques were experimentally infected intravenously with an attenuated strain of SIV, SIV/17E-Fr, and longitudinal serum samples were assayed to determine the extent of

SIV envelope-specific antibody maturation using a standard panel of serological assays [11]. Similar to the maturation of antibody responses described previously in rhesus macaques infected with closely related, highly attenuated SIV/17E-CL strain [8, 11], the SIV envelope-specific antibody response in these four monkeys demonstrated dramatic quantitative and qualitative changes during the first 6–8 months PI as measured by a standard panel of ELISA assays (Fig. 1). This gradual maturation of envelope-specific antibody responses has also been observed in other SIV systems [10, 13], and has been shown to be a common property of lentiviruses [10, 24, 31, 36]. All four animals quickly developed a high-titer envelope-specific response with endpoint titers at 12 months ranging from 2×10^4 to 4×10^5 (Fig. 1A). Similarly, all four animals progressed normally with regard to the qualitative measurements of avidity (Fig. 1B) and conformational dependence (Fig. 1C). By 8 months PI, all animals had reached an avidity index of approximately 50%, indicative of a mature antibody response. Additionally, all four monkeys displayed a gradual decline in conformational dependence, reaching a steady-state level by 8 months PI. One monkey, 0798, did display an increase in conformational dependence from 8 to 12 months, but the significance of this increase is not known. Finally, three of four monkeys exhibited a gradual steady increase in the ability of serum antibody to neutralize the closely related SIV/17E-CL virus. Antibodies from monkey 6998 fluctuated widely in their ability to neutralize virus. Initially, high neutralizing antibody titers were evident at 1 month PI. These titers decreased during months 2–4, rose rapidly again by month 5 and finally exhibited a slow, steady decline through month 12 PI. The results from these assays indicated that the infection of rhesus macaques with attenuated SIV/17E-Fr drives a maturation of the serum antibody response similar to infection with pathogenic SIV strains [10].

Binding characteristics of serum antibody as determined by SPR reveals dynamic antibody maturation process

In order to determine the binding characteristics of polyclonal serum antibodies using SPR, soluble envelope trimers were characterized. For these studies, SIV/17E-CL and SIVmac239 rgp140 antigens were expressed using a vaccinia expression system [17]; however, only the homologous SIV/17E-CL proteins were used in the binding studies. SIVmac239 was used as a reference in the event that SIV/17E-CL gp140 did not form stable trimers or bind CD4. SIV/17E-CL and SIV/17E-Fr are recombinant strains both derived from exchanging the gp120 (17E-CL) or gp120, gp41, nef and part of the 3' LTR (17E-Fr) from a primary brain-derived isolate of SIV into the SIVmac239 backbone [19]. Therefore, these two rgp140 antigens are >99% homologous. To characterize the multimeric nature of the SIVmac239 and SIV/17E-CL rgp140 proteins, both recombinant proteins were assayed on native polyacrylamide gels, transferred to membranes and probed with an envelope-specific MAb, 3.11H [42]. As seen in Fig. 2A, both recombinant proteins migrated as approximately 420 kDa homogenous proteins, consistent with findings seen by Center et al. [6, 7] demonstrating that secreted forms of SIV gp140 proteins are trimeric. Additionally, secreted recombinant proteins from both strains bound sCD4 at levels comparable with those observed with envelope from lysed virions (Fig. 2B).

The trimeric SIV/17E-CL rgp140s were used to determine the binding characteristics of polyclonal serum antibody from monkey 1998 using SPR analysis. Monkey 1998 was chosen because of the prototypical response observed using the standard ELISAs (Fig. 1). Additionally, monkey 1998 exhibited the highest titer anti-envelope antibody response among the four monkeys tested, as well as the highest peak and sustained viral load (Fig. 3). Serum samples were diluted, polyclonal IgG was captured, and binding experiments were carried out as described in the Materials and Methods section. Results from the SPR binding experiments are detailed in Table 1 and sensorgrams are shown in Fig. 4. In contrast to the steadily increasing avidity response seen in Fig. 1B, SPR analysis yielded a more dynamic

pattern of antibody evolution. At 1 month PI, there was one population of antibody that bound specifically to SIV/17E-CL rgp140 at an apparent association rate (k_{a1}) of 4.51×10^5 and apparent dissociation rate (k_{d1}) of 4.34×10^{-4} (Table 1). At 2 months PI, in addition to a population of antibody displaying similar apparent kinetics to that seen at 1 month ($k_{a1} = 5.19 \times 10^5$; $k_{d1} = 4.28 \times 10^{-5}$), we measured a second population of antibody. The two populations of antibody were present with equal magnitudes (R_{max}), however, the second population had relatively higher association rate ($k_{a2} = 3.43 \times 10^7$) and dissociation rate ($k_{d2} = 1.18 \times 10^{-2}$) values compared with the first population of antibody (k_{a1} and k_{d1}) (Table 1). To simplify further discussion, we refer to the first population observed (k_{a1} , k_{d1} , R_{max1}) as the ‘stable’ antibody population and the second antibody population as the ‘unstable’ antibody population, due to the relative differences in dissociation rate. Antibodies collected at 3 months PI again had one stable population of antibody that bound envelope with similar k_{a1} and k_{d1} values to the sample collected at 1 month PI, albeit with a higher magnitude (R_{max1}). Finally, antibodies collected at months 4, 5, 8, and 12 PI had both the stable and unstable antibody populations present (Table 1), with the stable antibody population comprising the higher percentage of the overall response (R_{max1}) at all four time-points. Together these data suggest the ability of SPR to define a more dynamic evolution of the polyclonal serum antibody response, particularly in the early time-points PI, than was measured using classical binding assays (Fig. 1).

Amino acid mutations in V1/V2 region of envelope over first 4 months PI

To determine if fluctuations in antibody populations observed by SPR analysis were related to an evolution of the viral envelope during the first months of infection, we amplified the V1/V2 sequence from proviral DNA extracted from serially sampled PBMC collected during the first 4 months PI by nested PCR. Mutations in the V1/V2 region emerged by 1 month PI (Fig. 5). However, no dominant secondary strain emerged. Interestingly, all the observed sequences had a loss of a potential N-linked glycosylation site at the beginning of V1 region that was observed at 2, 3, and 4 months PI. Further, at both 3 and 4 months PI, viruses incorporated a potential N-linked glycosylation site near the end of V1. However, one of the envelope clones at 4 months also demonstrated a loss of a potential N-linked glycosylation site resulting in a cancellation of the overall change in the number of glycosylation sites in this region (Fig. 5). Taken together, while there was no dramatic change in the envelope V1/V2 sequence during the first 4 months PI, mutations in this region that constitute minor variants did emerge that may be related to the dynamic changes in antibody populations observed by SPR.

Discussion

In this study we report dynamic changes in composition and binding characteristics of the SIV-specific polyclonal IgG response in experimentally infected rhesus macaques using SPR. These results were in marked contrast to the gradual maturation of envelope-specific antibody responses measured using a standard panel of serological binding assays. The current results suggest that the evolution of the antibody response is a more dynamic process than previously assumed, with the composition of the antibody response fluctuating between populations of high stability binding variously interspersed with a minor population of low binding stability. This finding has potential impact on the evaluation of vaccine strategies and may shed some light on the question of what constitutes an effective antibody response in SIV infection.

Classical assays to measure the quantity and quality of the antibody response during lentiviral infections include the titer, avidity, and conformation by ELISA (reviewed in [36]). In these assays, viral envelope proteins are captured by virtue of their extensive glycosylation onto microtiter plates by immobilized ConA, thereby better preserving the

conformation of the viral envelope compared with standard ELISAs where envelope proteins are directly adsorbed to the plastic of the ELISA plate [43]. Use of this modified ELISA protocol allows for the measurement of antibodies directed at conformational epitopes of the envelope protein. In addition, the stability of the ConA–envelope interaction is resistant to treatment with 8 M urea, thus enabling a measurement of the relative strength of antibody binding as the ability of the polyclonal antibody–envelope complex to resist treatment with 8 M urea (i.e., antibody avidity). These assays provide insight into the development of the antibody response in various lentiviral systems, including EIAV [15, 24, 31], FIV [31], SIV [10, 12, 13, 48], and HIV-1 [10, 39]. These studies in the SIV system in particular detailed the steady, continuous maturation of the polyclonal response from a low titer, low avidity, highly conformational response early in infection to one of high titer, high avidity and lowered conformational dependence 6–8 months PI when it reached a steady-state, mature level [10, 11]. Additionally, when developed to an attenuated strain of SIV, SIV/17E-CL, this matured immune response correlated with protection against pathogenic challenge at 8 and 28 months [11].

In contrast to the gradual maturation of antibody responses defined by these serological assays, binding characteristics as measured using SPR detailed a response that exhibited dynamic changes in the overall composition of the SIV-specific antibody response during the first 4 months PI that was not observed with the ELISA assays (Fig. 1). In addition, SPR assays provided apparent rates of antibody binding, a measure not previously possible with the serological assays used to define antibody maturation. This SPR-based assay allowed for the direct observation and measurement of two populations of polyclonal antibody that differed in terms of the apparent association and dissociation rates (Table 1). This response fluctuated in the early time-points (1–4 month PI) between a single population of antibody that bound SIV envelope in a stable manner at 1 month PI, the evolution of a second, unstable-binding population at 2 months PI in addition to the remaining stable population, the return to a single stable population at 3 months PI, and a final return to the two populations at 4 months PI that remained until 12 months PI. From 4 to 12 months PI, the two antibody populations differed only in the percentage response of stable/unstable populations to total response. Plotting the percentage of stable antibodies compared with the total measured response against the viral loads for animal 1998 demonstrated that the percentage of stable antibody seemed to act as a ‘magnification’ of the viral load, with both stable antibody and viral loads displaying similar trends (Fig. 6). Tracking the percentage change of the stable antibodies with concurrent changes in the plasma viral load may indicate that the presence and magnitude of the stable-binding antibody population occurs in response to viral evolution over time. Additionally, the inflection points in the stable antibody curve at 1, 2, 3, and 4 months PI may be indicative of an evolving immune response attempting to control the dominant viral population(s). The presence of a single stable-binding antibody population at 1 month PI is likely the initial response generated to the clonal input virus. The appearance of a second unstable-binding population may suggest the expansion of the humoral response in light of the emergence of phenotypically different viruses which were observed beginning at 2–4 months PI, i.e. the loss/gain of potential N-linked glycosylation sites observed in V1 (Fig. 5). The removal of N-linked glycosylation sites results in a redirection of the antibody response to regions in the V3 loop [13]. Further, the loss of the first glycosylation site (N at amino acid 114) in V1 corresponds closely to the loss of the glycosylation site with the greatest influence over redirection of the antibody response to the V3 loop [13]. This redirection of the immune response may be partially responsible for the appearance of an unstable-binding antibody population. Further studies are needed to determine the extent of evolution of the entire viral envelope. It will also be important to repeat these initial studies with additional monkeys to determine if this is an isolated phenomenon or a general characteristic of the antibody response.

This is the first time a dynamic evolution of the serum antibody responses during the first few months of an attenuated SIV infection in a rhesus macaque has been demonstrated in contrast to the previously defined antibody maturation profile. In particular, SPR-based binding studies determined the numerical descriptors for apparent rates of binding. These numerical descriptors indicated the presence of and allowed for the relative measurement of two major populations of antibody, one that bound the recombinant viral envelope protein with high stability and another that was unstable. Additionally, the percentage of the stable-binding antibodies tracked with fluctuations in the plasma viral load, indicating a potential link between antibody binding properties and the ability of virus to replicate. Finally, mutations observed in the V1/V2 region indicated changes in viral phenotype with respect to N-linked glycosylation sites that are known to cause redirection of the antibody response to the V3 loop. Taken together, this data demonstrates the effectiveness of Biacore technology in evaluating the antibody response during lentiviral infections.

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Fig. 1.

Antibody maturation profiles for SIV/17E-CL-infected rhesus macaques. Serum from rhesus macaques was tested for quantitative and qualitative reactivity to SIV envelope in a ConA ELISA (A–C) as well as its ability to neutralize homologous SIV/17E-CL (D). The maturation of the SIV-specific antibody response was indicative of the typical maturation seen in pathogenically infected animals [10] for (A) endpoint titer, (B) avidity index, and (C) conformation ratio. Neutralization of closely related SIV/17E-CL (D) increased steadily over the course of the 12 months examined.

Fig. 2. Characterization of SIV rgp140 trimers. Proteins were characterized as in Steckbeck et al. [47]. (A) Recombinant proteins from SIV/17E-CL and SIVmac239 were run on native polyacrylamide gel electrophoresis, and both proteins migrated to the expected 420 kDa. Monomeric gp120 also migrated as expected to 120 kDa. (B) Recombinant proteins were analyzed for the ability to bind sCD4 by ELISA. Both SIV/17E-CL and SIVmac239 gp120 bound sCD4 to levels similar to native viral envelope protein.

Fig. 3.

Rhesus macaque viral loads. Plasma viral loads were performed on serum from rhesus macaques to determine infection status and viral setpoint levels. All monkeys experienced peak viremia at 1 month PI. Monkeys 0798, 6998, and 7098 setpoint levels were $\sim 10^3$ RNA copies/ml plasma, while monkey 1998 had a setpoint of $\sim 10^4$ RNA copies/ml plasma.

Fig. 4.

Sensorgrams of surface plasmon resonance (SPR) analysis of 1998 samples. Serum samples from 1998 were run using SPR as stated in Materials and Methods. Sensorgrams provide an indication of binding (RU) over time (s) where association is visualized as an increasing response, during which SIV/17E-CL gp140 was flowed over the antibody, and dissociation is visualized as a decreasing response, during which gp140 was replaced with only buffer. Note similarity of 1- and 3-month time-points, where no sudden drop in response was observed during dissociation.

	V1							V2		
17EC1	RCN <u>K</u> SETDRW	GLTKSITTTA	STTSTTASAR	VDMVNETSSC	IAQDNCAGLE	QEQMISCKFN	MTGLNRDKKK	EYNETWYSAD	LVCEQGNNIG	
4	-----*	-----	-----	-----	-----I	-----	-----	-----	-----	(6/8)
4	-----	-----	-----	-----	-----I	-----R	-----	-----	-----Y	
4	-----	-----	-----	-----	-----I-S	-----	-----	-----	-----	
8	-----	-----	-----	-----	-----I	-----	-----	-----	-----	(6/11)
8	-----	-----	-----	-----	-----V	-----	-----	-----	-----	(2/11)
8	-d -----	-----	-----	-----P	-----I	-----	-----	-----	-----R	
8	-----	-----	-----	-----	-----D-I	-----	-----	-----	-----	
8	-----	-----	-----	-----	-----I	-----	-----	-----	-----S	
12	-----	-----	-----	-----	-----I	-----	-----	-----	-----	(5/10)
12	-----	-----	-----	-----	-----I	-----G	-----	-----	-----	(2/10)
12	-g -----	-----P	-----	-----	-----I	-----	-----	-----G	-----	
12	-----	-----	-----A	-----	-----T	-----	-----	-----	-----	
12	-----G	-----	-----	-----	-----I	-----	-----	-----	-----	
16	-----	-----	-----	-----	-----I	-----	-----	-----	-----	(6/10)
16	-----	-----	-----	-----	-----T	-----	-----	-----	-----	(2/10)
16	-d -----	-----	-----	-----	-----T	-----	-----	-----	-----	
16	-----	-----P	-----	-----	-----I	-----	-----	-----	-----	

Fig. 5.

Deduced amino acid sequence of the V1/V2 region of envelope aligned with SIV/17E-CL. The V1/V2 sequence was amplified as a measure of sequence evolution in envelope over the first 4 months PI. Potential N-linked glycosylation sites are underlined. The loss of a potential site is indicated by lower case bold lettering. Dashes represent identity to SIV/17E-CL, asterisk indicates a stop codon and a period is inserted to aid in alignment. Sequences were prepared for publication using SeqPublish (Los Alamos National Laboratory, <http://hiv-web.lanl.gov/content/index>).

Fig. 6.

Comparison of viral load and stable antibody in monkey 1998. Plasma viral loads were plotted against the percentage stable antibody population. Tracking of the trends may indicate an association between the level of virus in the plasma and the changes observed in antibody populations.

Table 1

Apparent rates of binding of polyclonal serum antibody populations

Sample	k_{a1} ($M^{-1} s^{-1}$)	k_{d1} (s^{-1})	R_{max1} (RU)	k_{a2} ($M^{-1} s^{-1}$)	k_{d2} (s^{-1})	R_{max2} (RU)
1-month	4.51×10^5	4.34×10^{-4}	2.5	N/A	N/A	N/A
2-month	5.19×10^5	4.28×10^{-5}	2.6	3.43×10^7	1.18×10^{-2}	3.0
3-month	2.93×10^5	3.16×10^{-4}	4.6	N/A	N/A	N/A
4-month	3.74×10^5	5.21×10^{-4}	7.7	3.91×10^7	9.93×10^{-3}	1.1
5-month	4.90×10^5	1.77×10^{-4}	10.6	8.27×10^7	1.47×10^{-2}	5.7
8-month	4.41×10^5	1.65×10^{-6a}	9.1	2.28×10^7	1.05×10^{-2}	5.8
12-month	3.37×10^5	1.44×10^{-7a}	13.1	4.32×10^6	3.76×10^{-2}	3.3

¹ R_{max} values for 1-, 2-, and 3-month samples are corrected to reflect the higher RU captured.

^a Values are extrapolated beyond the limits of detection for the Biacore 3000 and should be assumed to be 1×10^{-5} .