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High titer HIV-1 V3-specific antibodies with broad reactivity but low neutralizing potency in acute infection and following vaccination

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ABSTRACT

Identifying the earliest neutralizing antibody specificities that are elicited following infection or vaccination by HIV-1 is an important objective of current HIV/AIDS vaccine research. We have shown previously that transplantation of HIV-1 V3 epitopes into an HIV-2 envelope (Env) scaffold provides a sensitive and specific means to detect and quantify HIV-1 V3 epitope specific neutralizing antibodies (Nabs) in human sera. Here, we employ this HIV-2/HIV-1 V3 scaffolding strategy to study the kinetics of development and breadth of V3-specific Nabs in longitudinal sera from individuals acutely infected with clade C or clade B HIV-1 and in human subjects immunized with clade B HIV-1 immunogens. HIV-2/HIV-1 chimeras containing V3 sequences matched to virus type (HIV-2 or HIV-1), subtype (clade B or C), or strain (autologous or heterologous) were used as test reagents. We found that by 3–8 weeks post infection, 12 of 14 clade C subjects had a median IC_{50} V3-specific Nab titer of 1:700 against chimeric viruses containing a heterologous clade C V3. By 5 months post-infection, all 14 subjects were positive for V3-specific Nabs with median titers of 1:8000 against heterologous clade C V3 and 1:1300 against clade B V3. Two acutely infected clade B patients developed heterologous clade B V3-specific Nabs at titers of 1:300 and 1:1800 by 13 weeks of infection and 1:5000 and 1:11000 by 7 months of infection. Titers were not different against chimeras containing autologous clade B V3 sequences. Each of 10 uninfected normal human volunteers who were immunized with clade B HIV-1 Env immunogens, but none of five sham immunized control subjects, developed V3-specific Nabs titers as high as 1:3000 (median 1:1300; range 1:700–1:3000). None of the HIV-1 infected or vaccinated subjects had antibodies that neutralized primary HIV-1 virus strains. These results indicate that high-titer, broadly reactive V3-specific antibodies are among the first to be elicited during acute and early HIV-1 infection and following vaccination but these antibodies lack neutralizing potency against primary HIV-1 viruses, which effectively shield V3 from antibody binding to the functional Env trimer.

Introduction

Antibody specificities of the early humoral immune response to HIV-1 that contribute to virus containment are not fully understood. Antibody seroconversion occurs approximately 3 to 6 weeks following HIV-1 transmission and is characterized by the sequential appearance of plasma antibodies elicited against multiple viral proteins (Fiebig et al., 2003; Tomaras et al., 2008). Recent work has shown that the earliest antibody responses to HIV-1 are directed at the immunodominant region of the envelope (Env) protein gp41 and generally develop within 2 weeks after viral RNA (vRNA) is first detected in the plasma (Tomaras et al., 2008). Antibodies to Gag proteins arise at approximately 18 days (p24, p55) and 33 days (p17) following detection of plasma vRNA and antibodies targeting the polymerase proteins p66 and p31 appear approximately 21 and 53 days after detectable vRNA, respectively (Fiebig et al., 2003;

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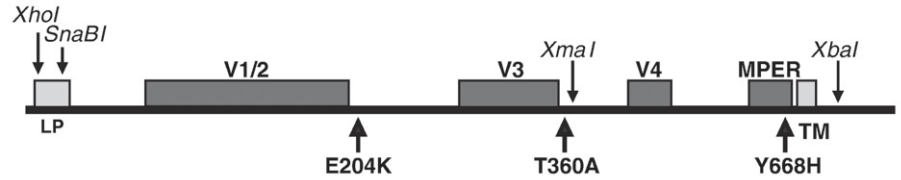
Tomaras et al., 2008). The Env glycoprotein gp120 elicits antibodies that can be identified in peptide and gp120 binding assays at approximately 28 days following detectable vRNA (Tomaras et al., 2008). The epitope specificities and breadth of reactivity of these early anti-gp120 antibodies have been the subject of limited investigation. A recent study mapped the earliest anti-gp120 binding antibody responses to include the third variable region (V3) and reported that antibodies specific for CD4-induced (CD4i) epitopes, the CD4 binding site (CD4bs), and the membrane proximal external region (MPER) of gp41 were not identified among early anti-Env responses (Tomaras et al., 2008). Additionally, antibodies targeting gp120 and gp41 during the first 40 days following detection of plasma vRNA did not exhibit neutralizing activity (Tomaras et al., 2008). This is in agreement with previous reports that documented the appearance of anti-gp120 binding antibodies prior to the development of autologous Nabs in the plasmas of acutely infected individuals (Aasa-Chapman et al., 2004; Moore et al., 1994). Antibodies capable of neutralizing the autologous virus strain develop later in infection, approximately 12–16 weeks following transmission, and such neutralizing antibodies (Nabs) are invariably strain specific (Frost et al., 2005; Gray et al., 2007; Richman et al., 2003; Wei et al., 2003). Early autologous Nab responses to HIV-1 generally target variable epitopes that are exposed on the functional Env trimer, namely V1, V2, and possibly V4, and drive the evolution of virus escape mutations that allow HIV-1 to rapidly evade Nab pressures (Frost et al., 2005; Honnen et al., 2007; McKeating et al., 1993; Moore et al., 2008; Pinter, 2007; Richman et al., 2003; Rong et al., 2007a,b; Wei et al., 2003). Only much later in infection, and only in a subset of HIV-1 infected individuals, do antibodies capable of mediating broad neutralization develop (Burton et al., 2004; Doria-Rose et al., 2009; Pantophlet and Burton, 2006; Sather et al., 2009). Recent studies have mapped these broad Nab responses to conserved gp120 epitopes including but not limited to the CD4bs (Binley et al., 2008; Dhillon et al., 2007; Li et al., 2007).

To identify what contributions V3-specific antibodies might make to the early Nab response to HIV-1 during acute and early infection and after immunization, we developed a novel assay for detecting and quantifying V3-specific Nabs in polyclonal human or animal sera. Our approach is based on the observation that, although the structure and functional biology of the HIV-1 and HIV-2 Envs are similar (Chen et al., 2005), the primary amino acid sequence varies by approximately 60% between these two glycoproteins and thus they are antigenically distinct at surface-accessible Nab epitopes (Bottiger et al., 1990; Decker et al., 2005; Weiss et al., 1988). In a previous study (Davis et al., 2009), we tested the hypothesis that substitution of the V3 region of HIV-2 with that of HIV-1 would result in a functional chimeric viral Env glycoprotein that could be employed as a molecular probe to detect V3-specific Nabs in polyclonal plasmas. We found such HIV-2/HIV-1 V3 chimeras to be infectious, replication competent, and sensitive to selective pharmacological inhibitors of virus entry. These chimeras were resistant to neutralization by HIV-1 monoclonal antibodies (mAbs) directed at the CD4bs (b12), membrane proximal external region (MPER; 2F5), and CD4-induced (CD4i) epitopes (E51, 17b, 48d, 21c, 4.12D, 19e, ED47, and ED49), but notably, were exquisitely sensitive to neutralization by HIV-1 V3-specific mAbs (447–52D and F425 B4e8) and HIV-1 V3-specific polyclonal antibodies in the plasma of chronically infected individuals (Davis et al., 2009).

Here, we explore the kinetics of appearance of V3-specific antibodies in a cohort of patients acutely infected with clade C or clade B HIV-1 and in subjects immunized with two vaccine products derived from clade B viruses. We explore the strain and subtype specificity of these V3-specific antibody responses and their contributions to autologous and heterologous virus neutralization. The results indicate that V3-specific antibodies develop faster and reach higher titers than do antibodies directed at CD4i, MPER, or CD4bs epitopes, and they are comparable or higher in titer compared with strain-specific Nab titers that typically develop in early infection (Gray et al., 2007; Li et al., 2006; Moore et al., 2008; Richman et al., 2003; Wei et al., 2003). However, despite the high titers and rapidity with which V3 antibodies are elicited, they contribute little, if any, neutralizing activity against autologous or heterologous primary viruses because the V3 epitopes of these viruses are effectively shielded from antibody access within the functional Env trimer. We discuss the potential importance of V3 Nab constraints on Env conformation and V3 accessibility in the native glycoprotein.

A



B

Virus	Backbone	Derivation	V3 Sequence	Adaptive Changes[a]
HIV-$2_{KR.X4}$	HIV-$2_{KR.P1}$	HIV-2_{KR}	CKRPGNKSVLPITLRSGRVFHSRPIINERPKQAWC	Absent
HIV-$2_{KR.X7}$	HIV-$2_{KR.P1}$	HIV-2_{KR}	CKRPGNKSVLPITLRSGRVFHSRPIINERPKQAWC	Present
HIV-$2_{KR.X7}$Ccon V3	HIV-$2_{KR.P1}$	HIV-1_{Ccon}	CTRPNNNTRKSIRIGPGQTFYATGDIIGDIRQAHC	Present
HIV-$_{2KR.X7}$YU2 V3	HIV-$2_{KR.P1}$	HIV-1_{YU2}	CTRPNNNTRKSINIGPGRALYTTGEIIGDIRQAHC	Present
HIV-$2_{KR.X7}$MN V3	HIV-$2_{KR.P1}$	HIV-1_{MN}	CTRPNYNKRKRIHIGPGRAFYTTKNIIGTIRQAHC	Present
HIV-$2_{KR.X7}$WEAU V3	HIV-$2_{KR.P1}$	HIV-1_{WEAU}	CIRPNNNTRKGITLGPGRVLYTTGEIIGDIRRAHC	Present
HIV-$_{2KR.X7}$BORI$_{d6}$ V3	HIV-$2_{KR.P1}$	HIV-1_{BORI_d6}	CTRPNNNTRKSIHIGPGRTFYTTGDIIGDIRQAYC	Present
HIV-$_{2KR.X7}$BORI$_{d556}$ V3	HIV-$2_{KR.P1}$	HIV-1_{BORI_d556}	CTRPNNNTRKGIHIGPGRTFYTTGDIIGNIRQAHC	Present

[a]Adaptive changes: E204K, T360A, and Y668H

Fig. 1. Description of HIV-$2_{KR.X7}$ Env scaffold and HIV-2/HIV-1 V3 chimeras. (A) The parental pHIV-$2_{KR.P1}$ *env* backbone (Davis et al., 2009) was modified to include unique silent restriction sequences in the leader peptide (LP) region (*XhoI* and *SnaBI*), the C3 coding region (*XmaI*), and 3′ of the transmembrane (TM) region (*XbaI*) and nonsynonymous mutations at amino acid positions 204 (E204K), 360 (T360A), and 668 (Y668H) to yield the final scaffold vector pHIV-$2_{KR.X7}$. V1/2, V3, V4 — variable regions 1/2, 3, and 4. MPER — membrane proximal external region. (B) V3 sequences for HIV-1_{MN}, HIV-1_{YU2}, HIV-1_{Ccon}, HIV-1_{WEAU}, HIV-1_{BORI_d6}, and HIV-1_{BORI_d556} were incorporated into the pHIV-$2_{KR.X7}$ *env* cassette as described in Materials and methods to generate the chimeric proviruses.

Table 1
Neutralization titers of clade C sera against HIV-2, HIV-1, and HIV-2/HIV-1 V3 chimeras.

Serum	WPI	Reciprocal IC_{50}					
		HIV-$2_{KR.X4}$	HIV-$2_{KR.X7}$	HIV-$2_{KR.X7}$ Ccon V3	HIV-$2_{KR.X7}$ YU2 V3	Autologous[a]	Heterologous (HIV-1_{DU151})
CAP008	3	<20	<20	845	53	<20	<20
	10	<20	<20	32425	1655	<20	<20
	19	<20	<20	>62500	4369	124	<20
	*25	<20	<20	>62500	5487	132	<20
	37	<20	<20	>62500	5122	401	<20
CAP030	5	<20	<20	675	71	ND[b]	<20
	*21	<20	<20	22880	3388	ND	<20
	29	<20	<20	24853	3355	ND	<20
	39	<20	<20	24471	4172	ND	<20
	46	<20	<20	27799	6539	ND	<20
	54	<20	<20	48207	11908	ND	<20
	58	<20	<20	30166	9259	ND	63
	71	<20	<20	39159	10256	ND	269
CAP045	5	<20	<20	<20	<20	<20	<20
	12	<20	<20	3609	436	56	<20
	18	<20	<20	3931	878	135	<20
	*26	<20	<20	2973	1472	261	<20
	35	<20	<20	2159	2950	512	<20
	43	<20	<20	3730	5519	5772	<20
CAP061	8	<20	<20	889	130	<20	<20
	15	<20	<20	2511	170	<20	<20
	*25	<20	<20	4278	214	<20	<20
	33	<20	<20	7536	528	<20	<20
	41	<20	<20	7690	198	<20	<20
	51	<20	<20	6813	254	38	104
	58	<20	<20	14675	407	74	100
	62	<20	<20	15059	210	61	111
CAP063	4	<20	<20	142	116	<20	<20
	11	<20	<20	5609	1603	<20	<20
	*20	<20	<20	17352	3707	642	<20
	29	<20	<20	19822	5454	5322	<20
	37	<20	20	21429	10958	6599	<20
CAP084	3	<20	<20	419	34	<20	<20
	10	<20	<20	1554	64	<20	<20
	*19	<20	<20	13317	636	<20	<20
	37	<20	<20	12193	1316	2360	<20
	47	<20	<20	13284	1719	1846	<20
CAP085	5	<20	<20	1485	121	<20	<20
	13	<20	<20	7673	182	40	<20
	*21	<20	<20	4579	1157	163	<20
CAP088	5	<20	<20	591	62	<20	<20
	13	<20	<20	5316	1476	<20	<20
	*22	<20	<20	8141	1182	656	<20
	30	<20	<20	21773	3285	1109	<20
	54	<20	<20	19542	3533	1363	<20
CAP206	8	<20	<20	15107	691	<20	<20
	15	<20	<20	28467	1360	96	<20
	*22	<20	<20	28946	2795	650	<20
CAP210	5	<20	<20	<20	<20	<20	<20
	12	<20	<20	20	<20	<20	<20
	18	<20	<20	1243	<20	<20	<20
	*26	<20	<20	3410	145	<20	<20
	38	<20	<20	4006	177	<20	<20
CAP228	7	<20	<20	2940	772	<20	<20
	14	<20	<20	11055	2884	51	<20
	*22	<20	<20	5374	2959	261	<20
	30	<20	<20	9705	3614	415	<20
	37	<20	<20	13668	4259	410	<20
CAP239	5	<20	<20	737	363	<20	<20
	11	<20	<20	6305	2626	<20	<20
	*19	<20	<20	3125	1420	331	<20
	29	<20	<20	5207	4832	708	<20
CAP244	8	<20	<20	1064	214	<20	<20
	14	<20	<20	2666	938	<20	<20
	*23	<20	<20	2809	618	88	<20
	32	<20	<20	2872	403	140	<20
	40	<20	<20	2781	321	274	<20
CAP255	8	<20	<20	598	54	<20	<20
	15	<20	<20	4778	<20	410	<20
	*23	<20	<20	8127	<20	3286	<20
	31	<20	<20	12766	146	2668	<20
	39	<20	<20	13547	601	2275	<20

[a] Autologous neutralization titers were obtained from Gray et al. (2007) for all sera.
[b] ND: not done as no cloned envelope was available from this individual.
* Indicates sera that were included in the analysis presented in Fig. 2B.

Results

Development of infectious HIV-2/HIV-1 V3 chimeras

Previously, we described the construction and biological characterization of a panel of HIV-2/HIV-1 V3 chimeras that detect V3 reactive antibodies in complex plasma with a high degree of sensitivity and specificity (Davis et al., 2009). These chimeras included the HIV-$2_{KR.X7}$ backbone (Fig. 1A) substituted with the V3 region of the HIV-1 strains HIV-1_{MN} (Gurgo et al., 1988), HIV-1_{YU2} (Li et al., 1992), HIV-1_{BORI_d6} (Keele et al., 2008; Wei et al., 2003) and HIV-1_{Ccon}. In the present study, we utilized all of these viruses plus two additional HIV-$2_{KR.X7}$HIV-1 V3 chimeras containing V3 loops from patients infected with clade B HIV-1, HIV-1_{WEAU} (Wei et al., 2003) and HIV-1_{BORI_d556} (Wei et al., 2003). The parental virus HIV-$2_{KR.X4}$, containing the wildtype HIV-2 Env protein, and the modified HIV-$2_{KR.X7}$ Env scaffold, that contains the wildtype HIV-2_{KR} V3 loop and three additional amino acid changes in Env that were found to enhance the infectivity of the HIV-2/HIV-1 V3 chimeras (Davis et al., 2009), were included as control viruses in all studies. Fig. 1B summarizes the composition of the HIV-2/HIV-1 V3 chimeras and control viruses.

Kinetics of appearance and breadth of V3 reactive antibodies during natural infection with clade C HIV-1

We assessed the kinetics of appearance of the V3-specific Nab responses in longitudinal serum samples from 14 subjects acutely infected with subtype C HIV-1 (CAPRISA cohort) using the HIV-$2_{KR.X7}$HIV-1 V3 chimeras and control viruses (Table 1 and Fig. 2A). All subjects produced V3-specific antibodies capable of neutralizing the clade-matched V3 chimera, HIV-$2_{KR.X7}$Ccon V3, within the first 12 weeks of infection. In 12 of 14 individuals, high-titer V3-specific antibodies were detectable in serum samples collected at enrollment into the study, which was estimated to be approximately 5 weeks (median, range of 3 to 8 weeks) post infection (wpi) (Gray et al., 2007). Enrollment sera neutralized the HIV-$2_{KR.X7}$Ccon V3 chimera at a median reciprocal IC_{50} serum dilution of 706 (range, <20 to 15,107) and increased in titer as infection progressed to as high as >62,500 (median = 13,608; range 2781 to >62,500). Additionally, the V3 neutralizing activities observed in these patients exhibited cross-clade reactivity as demonstrated by a median enrollment neutralization titer against the HIV-$2_{KR.X7}$YU2 V3 chimera of 94 (range, <20 to 772), which increased over time to as high as 10,958 (median = 3164; range 177 to 10,958). HIV-$2_{KR.X4}$ and HIV-$2_{KR.X7}$ served as controls for these experiments. Both viruses were resistant to neutralization by all sera, suggesting that neutralization of the HIV-$2_{KR.X7}$HIV-1 V3 chimeras was restricted to V3 epitopes (Table 1), which we confirmed by Fc-$V3_C$ fusion protein competition studies (Fig. 3).

The natural history of HIV-1 infection and sequential development of V3-specific Nabs in subject CAP008 highlight these observations (Fig. 2). Serum from CAP008 drawn at approximately 3 wpi neutralized the HIV-$2_{KR.X7}$Ccon V3 chimera at a reciprocal IC_{50} plasma dilution of 845. Subsequent serum samples from CAP008 neutralized the HIV-$2_{KR.X7}$Ccon V3 chimera with increasing potency (IC_{50} = 32,425 at week 10) reaching exceptionally high titers by week 19 post infection and beyond (>62,500 at weeks 19, 25, and 37).

These same sera neutralized the HIV-$2_{KR.X7}$YU2 V3 chimera at reciprocal IC_{50} plasma dilutions of 53 (week 3), 1655 (week 10), 4369 (week 19), 5487 (week 25), and 5122 (week 37). These observations were typical of most of the subjects participating in this natural history study and demonstrate both the rapid kinetics of appearance and high titers of V3 responses elicited by natural infection. In addition, they highlight a certain degree of clade specificity in what otherwise would be considered broad neutralizing activity (i.e., a Nab activity that neutralizes both clade C and clade B V3 chimeric viruses at titers exceeding 1:1000).

There were, however, exceptions to the pattern of neutralization found in subject CAP008. For example, CAP210 barely mounted a detectable V3-specific antibody response within the first 12 weeks of infection (Fig. 2A and Table 1); her serum at 12 wpi only weakly neutralized the clade-matched chimera (reciprocal IC_{50} plasma dilution = 20) and failed to neutralize the clade B (YU2) V3 chimera. At subsequent time points, the CAP210 V3-specific antibody response continued to increase in titer reaching a reciprocal IC_{50} titer of 4006 by 38 wpi. These antibodies, however, were of narrower breadth than those observed for other patients in this study as demonstrated by the slower kinetics and much lower titer of the reactivity to the HIV-$2_{KR.X7}$YU2 V3 chimera. Conversely, the breadth of the V3-specific antibody response observed for CAP045 and CAP239 was such that maximum Nab titers against the HIV-$2_{KR.X7}$Ccon V3 chimera and the HIV-$2_{KR.X7}$YU2 V3 chimera were equivalent (Fig. 2A and Table 1).

We next compared the V3-specific neutralizing titers of the CAPRISA sera measured against the HIV-2/HIV-1 chimeras with Nab titers measured against autologous and heterologous primary HIV-1 Envs. The autologous Envs were cloned from the enrollment plasma for each patient and used to pseudotype an Env-deficient HIV-1 backbone virus (Gray et al., 2007). Heterologous neutralization titers for the sera were determined against the clade C isolate HIV-1_{Du151}. For these studies, we plotted the reciprocal IC_{50} neutralization titers for sera that were obtained at a median time of 5 months post infection (range, 4.4 to 6.1) against the panel of HIV-$2_{KR.X7}$HIV-1 V3 chimeras and against autologous and heterologous HIV-1.

As demonstrated in Fig. 2B, all individuals in the CAPRISA natural infection cohort developed potent V3-specific Nab responses within the first 5 months of infection reaching a median reciprocal IC_{50} neutralization titer of 8127 (range, 2809 to >62,500) against the HIV-$2_{KR.X7}$Ccon V3 chimera. The cross-clade V3-specific reactivities were more variable and exhibited a median titer of 1301 (range, <20 to 5487) against the HIV-$2_{KR.X7}$YU2 V3 chimera at this time point. Neutralization of viruses pseudotyped with the autologous primary HIV-1 Env showed a median reciprocal IC_{50} serum dilution of 261 (range, <20 to 3286). This titer was significantly lower than either of the V3-specific Nab activities measured against the HIV-$2_{KR.X7}$Ccon V3 or HIV-$2_{KR.X7}$YU2 V3 chimeras ($p = 0.0162$ and 0.0285, respectively). These same sera demonstrated no detectable neutralization against a heterologous clade C primary Env, despite the preponderance of high titer V3-specific antibodies and detectable strain specific Nabs. These results are consistent with a report by Gray et al. (2007) in which it was shown that these same CAPRISA cohort sera obtained at 6 months post infection displayed limited neutralizing activity against other heterologous Envs. In our study, only two patients, CAP030 and CAP061, developed detectable Nabs against heterologous HIV-1_{Du151} within the first 58 weeks of infection, reaching maximum titers of 269 and 111, respectively (Table 1).

Epitope specificity of neutralization of HIV-$2_{KR.X7}$HIV-1 V3 chimeras by acute and early HIV-1^+ sera

To confirm the V3-specificity of neutralization detected by the HIV-$2_{KR.X7}$Ccon V3 chimera, we performed competition experiments using linear peptides composed of the full-length autologous V3 region or a fusion protein that presents the full-length Ccon V3 in its disulfide-bound form. For these competition studies, sera were incubated with peptide or fusion protein at 37°C 30 min prior to the addition of HIV-$2_{KR.X7}$Ccon V3 and transfer to the TZM-bl reporter cells. Fig. 3 shows that sequence-matched, full-length V3 peptides were relatively inefficient at removing the neutralizing activity in the CAPRISA sera: in all cases, these peptides absorbed only a fraction of the serum neutralizing activity detected by the HIV-$2_{KR.X7}$Ccon V3 chimera as evidenced by a rightward shift in the neutralization curves. For example, serum from CAP084 drawn at enrollment neutralized HIV-$2_{KR.X7}$Ccon V3 at a reciprocal IC_{50} plasma dilution of 423 in the absence of competing peptide. Pre-incubation of CAP084 serum with autologous V3 peptide resulted in a reciprocal IC_{50} titer of 179 against the HIV-$2_{KR.X7}$Ccon V3 chimera, a modest (<3-fold) reduction in the V3-specific neutralizing activity of this serum. Linear peptide absorption of V3-specific antibodies from CAP206 serum was more effective and resulted in an approximately 10-fold reduction in the serum neutralizing titer against HIV-$2_{KR.X7}$Ccon V3.

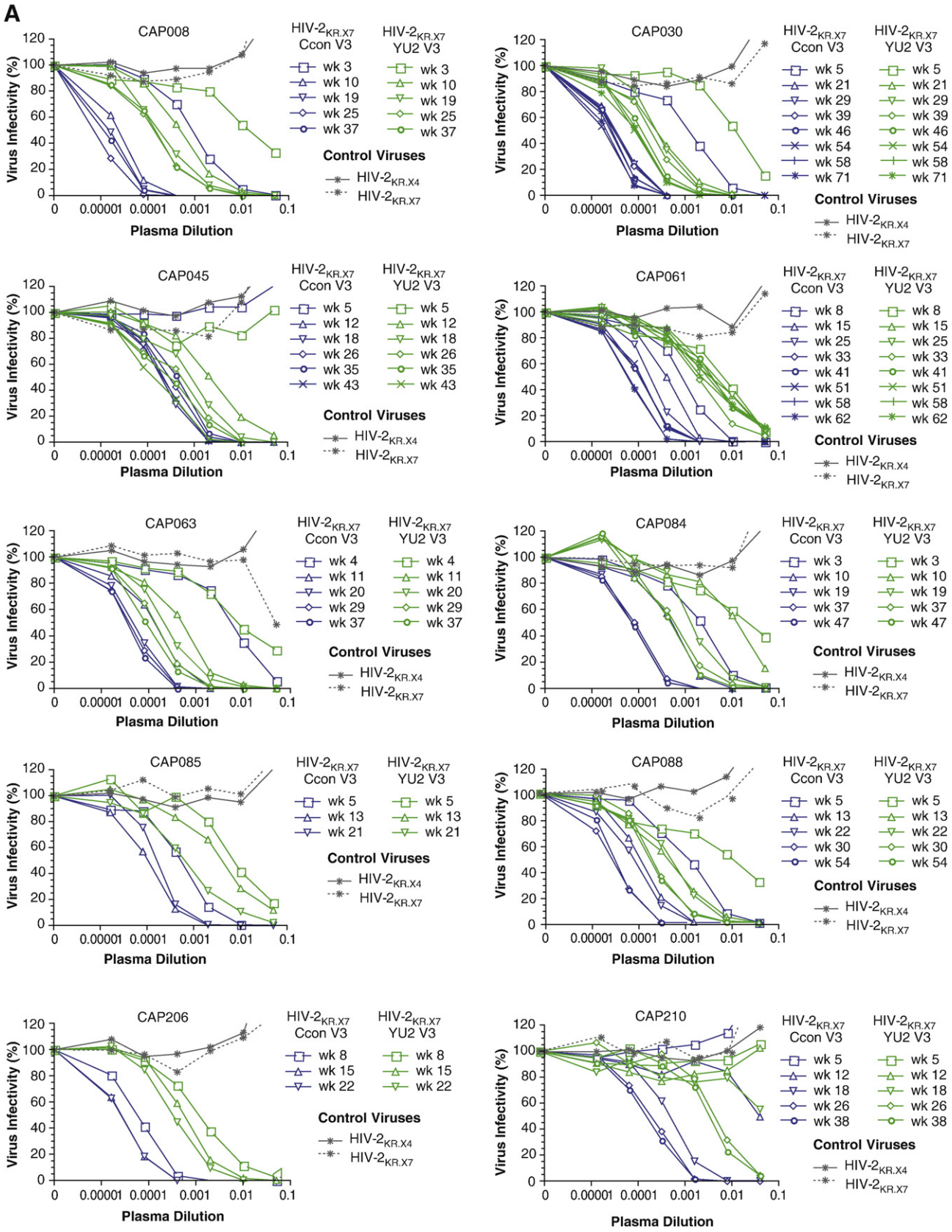
Because linear peptides were inefficient at competing with the HIV-$2_{KR.X7}$Ccon V3 chimera for serum antibody binding, we hypothesized that the linear peptides might not replicate V3 secondary and tertiary structures sufficiently to serve as effective competing antigens. We therefore conducted similar absorption experiments using a chimeric fusion protein containing a rabbit Fc backbone with the full, disulfide bound HIV-1_{Ccon} V3 fused to its amino terminus, referred to as Fc-$V3_C$ FP (A.P. and A.S., unpublished, and Davis et al., 2009). This reagent was shown in prior studies to compete specifically and far more effectively than linear V3 peptides against V3-specific mAbs and V3-specific plasma antibodies from chronically infected subjects (Davis et al., 2009). In all samples tested, pre-incubation with Fc-$V3_C$ FP completely removed the serum neutralizing activity, verifying that the HIV-$2_{KR.X7}$Ccon V3 chimera is detecting only antibodies directed at the V3 loop and not reactivity at other HIV-2 epitopes. Neither a scrambled V3 peptide nor a rabbit Fc protein lacking the V3 loop depleted the neutralizing activities of V3-specific mAbs (447-52D and F425 B4e8) or plasma antibodies from infected subjects, thus confirming that V3 peptides and the rabbit Fc-$V3_C$ FP do not compete nonspecifically for plasma antibody reactivity (Davis et al., 2009).

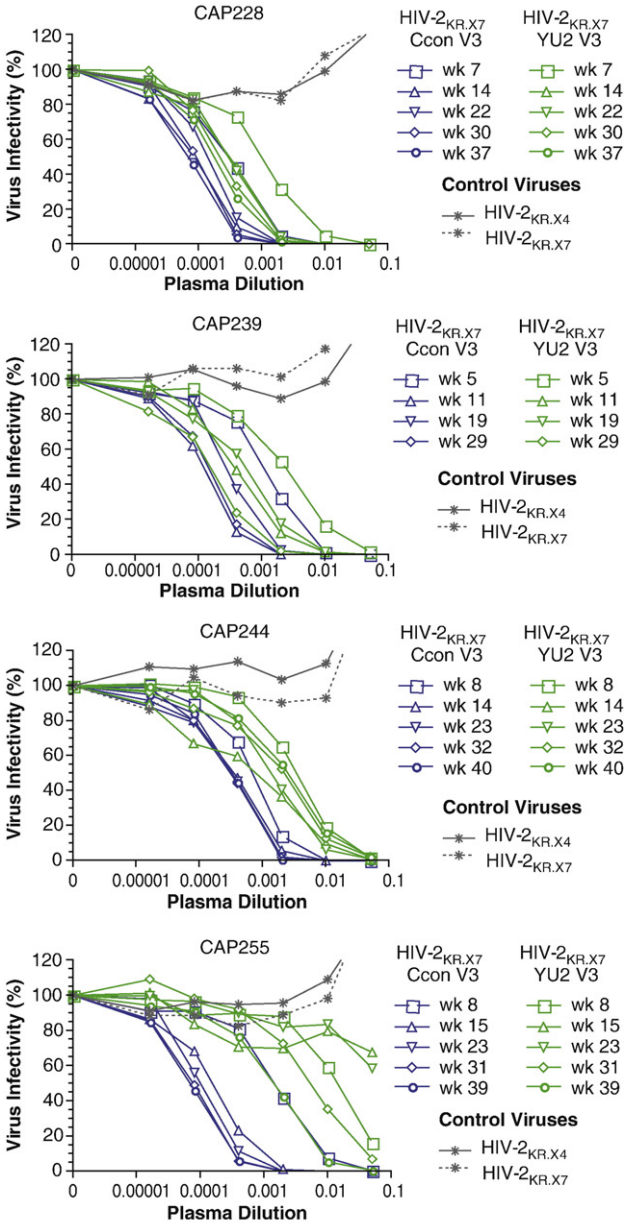
Kinetics of appearance and breadth of V3 reactive antibodies during natural infection with clade B HIV-1

We performed similar analysis using longitudinal plasma samples obtained from two patients acutely infected with clade B HIV-1. Data presented in Fig. 4 and Table 2 show the kinetics of appearance of V3-specific antibodies following clade B HIV-1 infection. Plasma samples obtained from subject WEAU at days 16, 20, 30, 40, and 51 post onset of symptoms (corresponding to 36, 40, 50, 60, and 71 days after the estimated date of infection) contained no detectable V3-specific antibodies (Fig. 4A). V3-specific antibody responses were detected by day 72 post onset of symptoms and increased in titers thereafter, reaching a maximum IC_{50} neutralizing titer of 5275 by day 212 against the clade-matched chimera, HIV-$2_{KR.X7}$YU2 V3. Similar to our findings with the clade C natural infection cohort, cross-clade V3-specific neutralizing titers for subject WEAU were lower, reaching a maximum IC_{50} plasma dilution of 276 against the HIV-$2_{KR.X7}$Ccon V3 chimera by day 212 post onset of symptoms. We made similar observations for subject BORI who was also sampled during acute infection by clade B HIV-1 (Fig. 4B and Table 2). V3-specific antibodies capable of neutralizing the HIV-$2_{KR.X7}$YU2 V3 chimera were detected in plasma obtained on day 65 after the onset of symptoms (approximately 79 days after the estimated date of infection) and increased in potency to an IC_{50} neutralizing titer of 28,729 by day 556. These antibodies again demonstrated diminished but readily detectable cross-clade

reactivity with IC_{50} titers against the HIV-$2_{KR.X7}$Ccon V3 chimera of 2066 by day 556 post onset of symptoms.

We considered the possibility that within-clade V3 diversity might limit our ability to detect clade B V3-specific responses in the earliest plasma specimens since we had used an HIV-2/HIV-1 V3 Env chimera containing a heterologous B clade V3 sequence from a prototypic strain HIV-1_{YU2} (Davis et al., 2009). We therefore constructed three additional HIV-$2_{KR.X7}$HIV-1 V3 chimeras each containing the V3 loop





autologous to that of virus amplified from the subjects WEAU and BORI. The V3 sequences for the HIV-$2_{KR.X7}$WEAU V3 and HIV-$2_{KR.X7}$BORI V3 chimeras are presented in Fig. 1B. Fig. 4A and Table 2 show that for patient WEAU, plasma V3-specific neutralizing activity directed at autologous V3 epitopes, measured by the HIV-$2_{KR.X7}$WEAU V3 chimera, develops with the same kinetics and potency as that directed at within-clade heterologous epitopes present on HIV-$2_{KR.X7}$YU2 V3, and that the neutralization curves for these viruses are superimposable. The results were similar for BORI. Two chimeras containing V3 sequences autologous to HIV-1_{BORI} were used in these experiments. HIV-$2_{KR.X7}$BORI$_{d6}$ V3 contains the V3 loop of virus amplified on day 6 post onset of symptoms and HIV-$2_{KR.X7}$BORI$_{d556}$ V3 contains the V3 loop of day 556 virus. Neutralization curves of HIV-$2_{KR.X7}$BORI$_{d6}$ V3, HIV-$2_{KR.X7}$BORI$_{d556}$ V3, and HIV-$2_{KR.X7}$YU2 V3 by serial BORI plasmas were again virtually superimposable (Fig. 4B and Table 2). These results highlight the antigenic conservation of V3 that exists within clade B HIV-1 and suggest that the earliest V3 Nab responses in natural infection exhibit substantial heterologous within-clade cross reactivity.

Titers and breadth of V3 antibodies elicited by vaccination

We next sought to assess the breadth and titers of V3-specific Nabs elicited after immunization with two HIV-1 vaccine candidates and determine to what degree V3 antibodies might contribute to the neutralizing potency of the sera against a heterologous primary virus, HIV-1_{YU2}. In the HVTN Protocol 203 vaccine study, participants were immunized with a canarypox HIV vaccine, ALVAC-HIV vCP1452, that encodes the HIV-1_{MN} gp120, full HIV-1_{LAI} Gag protein, a portion of the *pol* gene, and multiple CTL epitopes located in *nef* and *pol*. ALVAC-HIV vCP1452, or a sham control, was administered by intramuscular injection to all participants and responses were boosted in a subset of individuals with the recombinant AIDSVAX™ B/B gp120 subunit immunogen composed of the gp120 proteins from HIV-1_{MN} and HIV-1_{GNE8} (both derived from subtype B viruses). A detailed description of the HVTN Protocol 203 vaccine cohort and immunization schedule has been published (Russell et al., 2007).

Our analysis represented a pilot study designed to test if V3-specific antibodies could be detected in vaccinees. Power calculations indicated that ten-fold differences in median IC_{50} titers, if present, could be detected between groups of five subjects given the precision and reproducibility of the TZM-bl neutralization assay (Montefiori, 2004; Wei et al., 2003). The analyses were thus conducted on a subset of HVTN Protocol 203 study participants that included five subjects in each of three immunization groups: those receiving four immunizations with the ALVAC-HIV vCP1452 immunogen alone, those receiving four immunizations with the ALVAC-HIV vCP1452 immunogen plus AIDSVAX protein boosts, and those receiving four immunizations with a placebo immunogen. We tested sera from these patients for V3-specific neutralizing activity against an HIV-2/HIV-1 V3 chimera containing the autologous HIV-1_{MN} V3 loop (HIV-$2_{KR.X7}$MN V3) and

Fig. 2. Kinetics of development and breadth of V3-specific antibodies during acute clade C HIV-1 infection. (A) HIV-$2_{KR.X7}$HIV-1 V3 chimeras and control viruses were tested for neutralization susceptibility to sera obtained from fourteen patients acutely infected with clade C HIV-1 (CAPRISA cohort) (Gray et al., 2007; Moore et al., 2008). Virus entry was measured by luciferase production in TZM-bl reporter cells 48 h after infection and normalized to luciferase expression in the absence of serum. Neutralization of HIV-$2_{KR.X7}$Ccon V3 is represented by blue lines. Neutralization of HIV-$2_{KR.X7}$YU2 V3 is shown in green. Neutralization of control viruses, HIV-$2_{KR.X4}$ and HIV-$2_{KR.X7}$, by serum drawn at the last time point for each patient, is shown in gray. IC_{50} neutralization values are presented in Table 1. (B) Reciprocal IC_{50} neutralization titers for CAPRISA sera against HIV-$2_{KR.X7}$Ccon V3 (blue), HIV-$2_{KR.X7}$YU2 V3 (green), autologous clade C HIV-1 (black), and the heterologous clade C HIV-1_{Du151} (gray), are plotted. Sera for these studies were obtained at approximately 5 months post infection for each patient. Median values are marked with a horizontal line. IC_{50} values for all sera tested against control HIV-2 viruses were <1:20. Comparisons that show statistical significance are indicated.

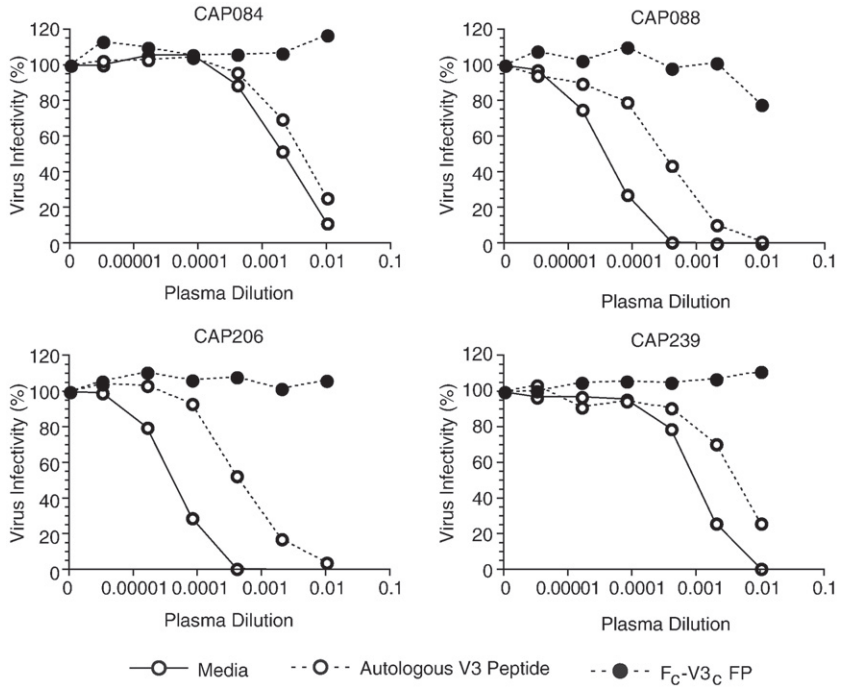


Fig. 3. Epitope specificity of neutralization of HIV-$2_{KR.X7}$Ccon V3 chimeras by clade C sera. Neutralization of the HIV-$2_{KR.X7}$Ccon V3 chimera by sera obtained from CAP084, CAP088, CAP206, and CAP239 is inhibited modestly by full-length linear V3 peptides matching the autologous sequence (dashed line, open symbol) and completely by the Fc-$V3_C$ fusion protein (FP; dashed line, closed symbol). Media only controls (solid line, open symbol) show serum neutralization in the absence of competing peptide or Fc-$V3_C$ FP. Neither a scrambled V3 peptide nor a rabbit Fc protein lacking the V3 loop depleted the neutralizing activities of clade C plasma antibodies, thus confirming that V3 peptides and the rabbit Fc-$V3_C$ FP do not compete nonspecifically for plasma antibody reactivity (data not shown and Davis et al., 2009).

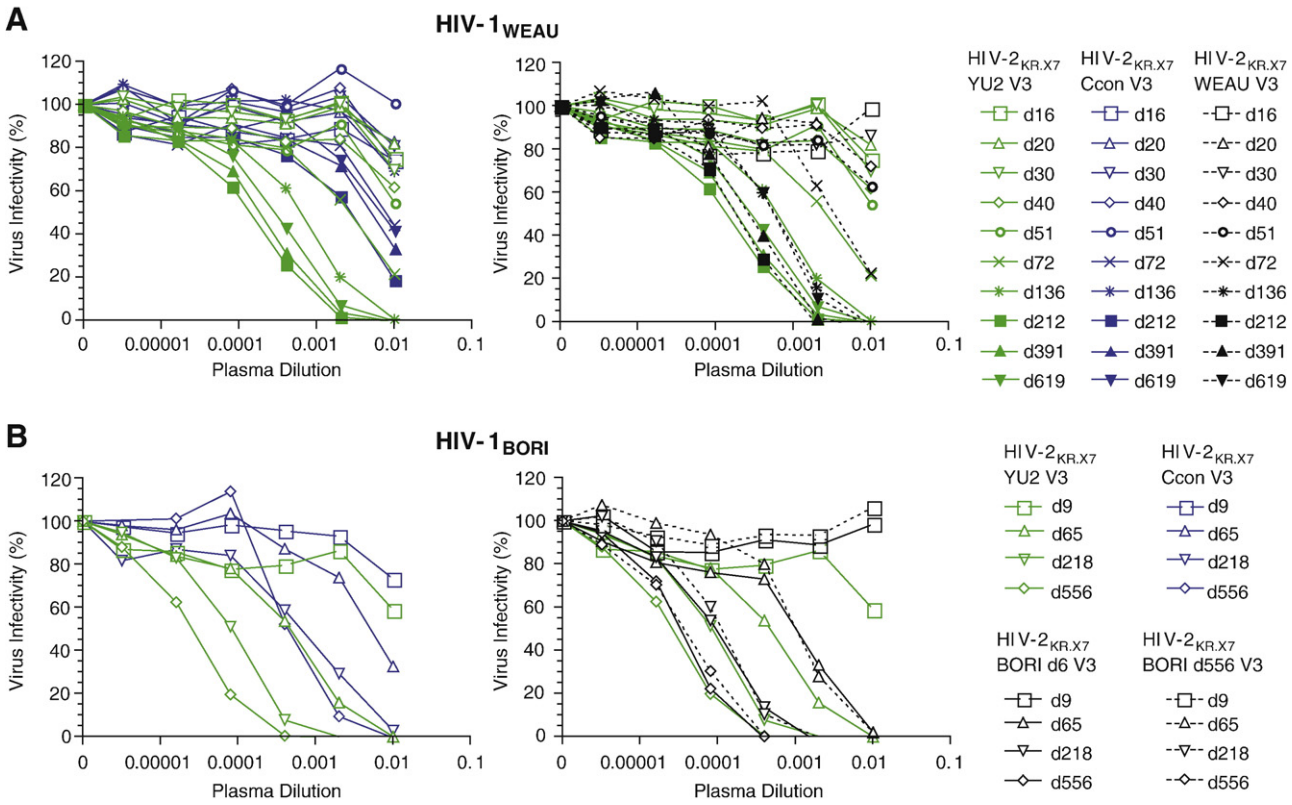


Fig. 4. Kinetics of development and breadth of V3-specific antibodies during acute clade B HIV-1 infection. HIV-$2_{KR.X7}$HIV-1 V3 chimeras and control viruses were tested for neutralization sensitivity to plasma obtained from two patients acutely infected with clade B HIV-1. Neutralization of HIV-$2_{KR.X7}$YU2 V3 (green) and HIV-$2_{KR.X7}$Ccon V3 (blue) by plasma samples from each time point is shown. To assess the effects of within-clade V3 diversity on the detection of V3-specific antibodies by the HIV-$2_{KR.X7}$HIV-1 V3 chimeras, we constructed three additional chimeras containing V3 sequences matching those of patients WEAU and BORI. (A) Neutralization of HIV-$2_{KR.X7}$WEAU V3 by autologous plasmas is shown in black. (B) Neutralization of HIV-$2_{KR.X7}$HIV-1 V3 chimeras containing the V3 sequence of HIV-1_{BORI} from day 6 and day 556 post onset of symptoms by autologous plasmas is shown in black (solid lines: HIV-$2_{KR.X7}BORI_{d6}$ V3; dashed lines: HIV-$2_{KR.X7}BORI_{d556}$ V3). IC_{50} neutralization values are presented in Table 2.

Table 2
Neutralization titers of clade B plasmas against HIV-2, HIV-1, and HIV-2/HIV-1 V3 chimeras.

Plasma	Days[a]	Reciprocal IC_{50}						
		HIV-$2_{KR.X4}$	HIV-$2_{KR.X7}$	HIV-$2_{KR.X7}$ YU2 V3	HIV-$2_{KR.X7}$ Ccon V3	HIV-$2_{KR.X7}$ WEAU V3	HIV-$2_{KR.X7}$ $BORI_{d6}$ V3	HIV-$2_{KR.X7}$ $BORI_{d556}$ V3
WEAU	16	<100	<100	<100	<100	<100	ND	ND
	20	<100	<100	<100	<100	<100	ND	ND
	30	<100	<100	<100	<100	<100	ND	ND
	40	<100	<100	<100	<100	<100	ND	ND
	51	<100	<100	<100	<100	<100	ND	ND
	72	<100	<100	292	<100	216	ND	ND
	136	<100	<100	1179	114	1310	ND	ND
	212	<100	<100	5275	276	4097	ND	ND
	391	<100	<100	4061	152	3119	ND	ND
	619	<100	<100	2978	125	1345	ND	ND
BORI	9	<100	<100	<100	<100	ND[b]	<100	<100
	65	<100	<100	1790	150	ND	756	756
	218	<100	<100	11282	1145	ND	9038	6868
	556	<100	<100	28729	2066	ND	22605	20586

[a] Days post onset of symptoms.
[b] ND: Not done.

against chimeras containing heterologous V3 loop sequences, HIV-$2_{KR.X7}$YU2 V3 and HIV-$2_{KR.X7}$Ccon V3. The control viruses HIV-$2_{KR.X4}$ and HIV-$2_{KR.X7}$ were also tested with all sera. Results are summarized in Fig. 5 and Table 3. Fig. 5A shows that all subjects receiving the ALVAC-HIV vCP1452 immunogen developed moderately high titer V3-specific antibodies. These antibodies recognized the autologous MN V3 loop most efficiently, the heterologous clade B YU2 V3 loop intermediately, and the cross-clade subtype C V3 sequence least well. For example, subject ALVAC1, who received four immunizations with the ALVAC-HIV vCP1452 immunogen alone, developed V3-specific antibodies that neutralized the autologous HIV-$2_{KR.X7}$MN V3 chimera at a reciprocal IC_{50} serum dilution of 906, the heterologous HIV-$2_{KR.X7}$YU2 V3 chimera at a reciprocal IC_{50} of 136, and the cross-clade heterologous HIV-$2_{KR.X7}$Ccon V3 chimera at a titer of 111. Fig. 5D shows this to be a general feature of the V3 response to the ALVAC-HIV vCP1452 immunogen. Median reciprocal IC_{50} neutralization titers for all five subjects were 1,160 (range, 906 to 1745) against HIV-$2_{KR.X7}$MN V3 and less against HIV-$2_{KR.X7}$YU2 V3 (IC_{50} = 156; range, 68 to 802; $p = 0.0006$) and HIV-$2_{KR.X7}$Ccon V3 (IC_{50} = 142; range, 23 to 402; $p = 0.0009$). These data demonstrate that vaccination with the ALVAC-HIV vCP1452 immunogen alone elicits V3-specific antibodies of substantial titer but limited breadth.

The V3 antibody responses elicited by the ALVAC-HIV vCP1452/AIDSVAX immunization schedule, which included an Env protein boost, were more substantial and showed much greater breadth of V3 reactivity (Fig. 5B). All five subjects who received four immunizations with the ALVAC-HIV vCP1452 immunogen plus gp120 protein boosts developed high titer V3-specific antibodies that neutralized the HIV-$2_{KR.X7}$MN V3, HIV-$2_{KR.X7}$YU2 V3, and HIV-$2_{KR.X7}$Ccon V3 chimeras at equivalent titers. For these subjects, the median reciprocal IC_{50} serum dilutions were 1527 against HIV-$2_{KR.X7}$MN V3 (range, 710 to 3078), 3137 against HIV-$2_{KR.X7}$YU2 V3 (range, 883 to 3338), and 1997 against HIV-$2_{KR.X7}$Ccon V3 (range, 497 to 2307) (Fig. 5D). The control viruses, HIV-$2_{KR.X4}$ and HIV-$2_{KR.X7}$, were not neutralized by sera from vaccines immunized with either ALVAC-HIV vCP1452 or ALVAC-HIV vCP1452/AIDSVAX sera, indicating that the observed neutralizing activities in the sera against the HIV-$2_{KR.X7}$HIV-1 V3 chimeras were directed at HIV-1 V3 epitopes. Pre-immune sera from ALVAC-HIV vCP1452 and ALVAC-HIV vCP1452 plus AIDSVAX study participants (Figs. 5A–C), and from those receiving a placebo immunogen (Fig. 5C), failed to neutralize the HIV-$2_{KR.X7}$HIV-1 V3 chimeras or the control viruses.

We also assessed the ability of vaccinee sera to neutralize autologous and heterologous HIV-1 viruses. Autologous neutralization was measured against the laboratory-adapted, neutralization sensitive clade B HIV-1_{MN} virus, a component of the vaccine formulation for both the ALVAC-HIV vCP1452 and ALVAC-HIV vCP1452/AIDSVAX groups. Serum neutralization of HIV-1_{YU2} served as a measure of heterologous neutralization for these experiments. Fig. 5D summarizes the results. Sera from individuals receiving the ALVAC-HIV vCP1452 immunogen alone displayed weak neutralizing activity against HIV-1_{MN} (reciprocal IC_{50} serum dilution = 56; range, 56 to 100) at titers that were significantly lower than those observed for the HIV-$2_{KR.X7}$MN V3 chimera ($p = 0.0016$). None of the subjects immunized with the ALVAC-HIV vCP1452 vaccine developed Nabs against the heterologous HIV-1 clade B strain, HIV-1_{YU2}. Sera from individuals receiving the ALVAC-HIV vCP1452 vaccine plus AIDSVAX rgp120 protein boost neutralized the autologous HIV-1 strain, HIV-1_{MN}, at slightly higher titers (median reciprocal IC_{50} = 100; range, 91 to 139), but again these titers were significantly lower than those measured against the HIV-$2_{KR.X7}$MN V3 chimera ($p = 0.0148$), the HIV-$2_{KR.X7}$YU2 V3 chimera ($p = 0.0107$), and the HIV-$2_{KR.X7}$Ccon V3 chimera ($p = 0.0184$). None of the vaccinee sera, which potently neutralized the three HIV-2/HIV-1 V3 chimeras, displayed detectable neutralization against the primary HIV-1_{YU2} virus (IC_{50}<20; p<0.05 for each).

Discussion

Deciphering the specificities of the earliest Nabs that arise during natural HIV-1 infection and in response to vaccination and defining their contributions to constraints on HIV-1 replication and evolution can contribute to our understanding of HIV-1 natural history, and eventually, to effective vaccination design. Previous studies of the antibody response directed to HIV-1 V3 in chronically infected humans and in vaccinated animals demonstrated the exceptional immunogenicity of V3 peptide sequences *in vivo* (Davis et al., 2009; Goudsmit et al., 1988; Javaherian et al., 1989; Nara et al., 1990; Palker et al., 1988; Profy et al., 1990; Rusche et al., 1988; Wu et al., 2006; Zolla-Pazner et al., 2008), but the kinetics of appearance of V3-specific Nabs were far less well characterized (Goudsmit et al., 1988; Moore et al., 1994; Tomaras et al., 2008). In addition, the breadth of reactivity of early V3 Nab responses and their contributions to neutralization of primary virus strains in acute infection or following vaccination had not been clearly elucidated. In the present study, we employed a panel of HIV-2/HIV-1 V3 chimeras to quantify V3-specific Nabs in sera from individuals acutely infected with clade C or clade B HIV-1 and from subjects vaccinated with two immunogens derived from clade B HIV-1 strains. We found that during acute infection with clade C or clade B HIV-1, patients rapidly develop V3-specific antibodies that affect potent neutralization of the HIV-$2_{KR.X7}$HIV-1 V3 chimeras. As early as

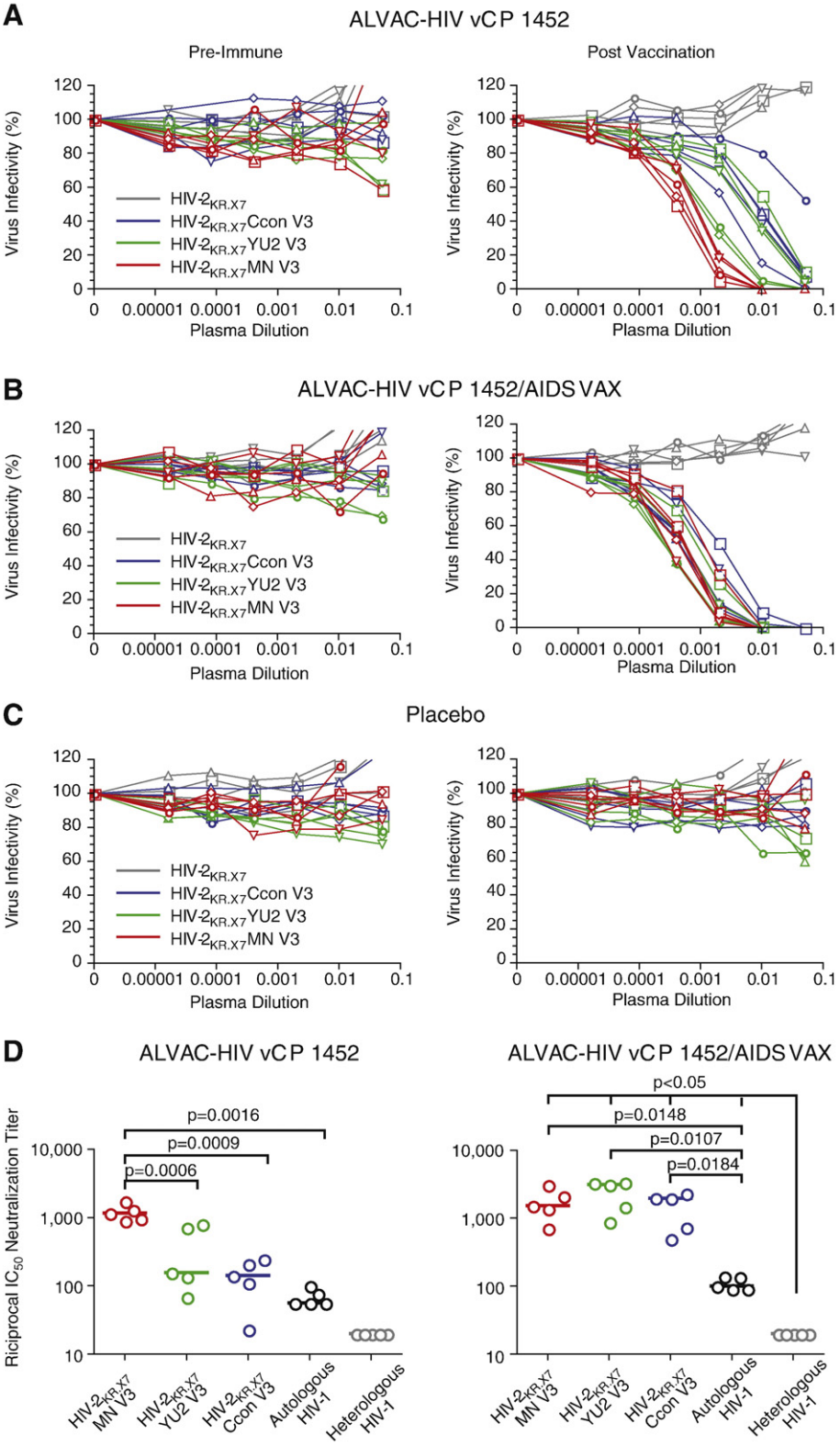


Fig. 5. Breadth of V3-specific antibodies elicited by vaccination with HIV-1 immunogens. Sera from individuals immunized with ALVAC-HIV vCP1452 vaccine (A), ALVAC-HIV vCP1452 and AIDSVAX rgp120 B/B protein (B), or placebo (C) were tested for neutralizing activity against the HIV-$2_{KR.X7}$HIV-1 V3 chimeras and control viruses. Virus neutralization by paired pre-immune sera is shown in the left panels. Neutralization of HIV-$2_{KR.X7}$MN V3, containing the V3 loop autologous to that of the vaccine immunogens, is shown in red. Neutralization of HIV-$2_{KR.X7}$YU2 V3 and HIV-$2_{KR.X7}$Ccon V3 is shown in green and blue, respectively. Neutralization of the control virus HIV-$2_{KR.X7}$ is shown in gray. IC_{50} neutralizing titers are reported in Table 3. (D) Reciprocal IC_{50} neutralization titers for vaccinee sera against HIV-$2_{KR.X7}$MN V3 (red), HIV-$2_{KR.X7}$YU2 V3 (green), HIV-$2_{KR.X7}$Ccon V3 (blue), autologous clade B virus (HIV-1_{MN}; black), and the heterologous clade B virus (HIV-1_{YU2}; gray), are plotted. Median values are marked with a horizontal line. IC_{50} values for all sera tested against control HIV-2 viruses was <1:20. Comparisons that show statistical significance are indicated.

Table 3
Neutralization titers of vaccine sera against HIV-2, HIV-1, and HIV-2/HIV-1 V3 chimeras.

Subject	Reciprocal IC_{50}						
	HIV-$2_{KR.X4}$	HIV-$2_{KR.X7}$	HIV-$2_{KR.X7}$ MN V3	HIV-$2_{KR.X7}$ YU2 V3	HIV-$2_{KR.X7}$ Ccon V3	Autologous (HIV-1_{MN})	Heterologous (HIV-1_{YU2})
ALVAC							
1	<20	<20	906	136	111	56	<20
2	<20	<20	1160	68	246	56	<20
3	<20	<20	968	156	142	77	<20
4	<20	<20	1745	802	402	56	<20
5	<20	<20	1307	714	23	100	<20
ALVAC/AIDSVAX							
1	<20	<20	710	883	497	100	<20
2	<20	<20	1527	3137	2307	139	<20
3	<20	<20	3078	1478	733	91	<20
4	<20	<20	2121	3338	1977	91	<20
5	<20	<20	1388	3295	1993	237	<20
Placebo							
1	<20	<20	<20	<20	<20	<20	<20
2	<20	<20	<20	<20	<20	<20	<20
3	<20	<20	<20	<20	<20	<20	<20
4	<20	<20	<20	<20	<20	<20	<20
5	<20	ND[a]	<20	<20	<20	<20	<20

[a] ND: Not done.

approximately 5 wpi, sera from 12 of 14 (86%) clade C HIV-1 infected subjects (CAPRISA cohort) neutralized the clade-matched V3 chimera, HIV-$2_{KR.X7}$Ccon V3, at a median reciprocal IC_{50} serum dilution of 706 (Fig. 2A and Table 1). All of these subjects exhibited V3-specific neutralizing activities by 12 wpi, which was prior to or coincident with the development of autologous Nabs for these individuals (Table 1). By 5 months post infection, clade C infected subjects developed V3-specific antibodies that reached a median IC_{50} neutralizing titer of 8127. These latter results are similar to our findings in a previous study of plasmas from individuals chronically infected with clade C HIV-1 in which we found that neutralizing titers against HIV-$2_{KR.X7}$Ccon V3 occurred at a median reciprocal IC_{50} plasma dilution of 8488 (Davis et al., 2009). Also in agreement with our findings in chronically infected subjects, we found that the anti-V3 reactivities detected in subjects acutely by clade C virus patients could neutralize the HIV-$2_{KR.X7}$YU2 V3 chimera, containing a clade B HIV-1 V3 loop. These cross-clade V3-specific neutralizing titers were detected at enrollment and increased as infection progressed.

A comparison of the kinetics of appearance of V3-specific Nabs with those targeting CD4i epitopes, the MPER, or other epitopes that mediate autologous neutralization (Davis et al., 2009; Gray et al., 2007; Moore et al., 2008) demonstrated that anti-V3 Nabs are the first of this group to appear during acute clade C HIV-1 infection. For subjects in the CAPRISA natural infection cohort, antibodies directed at CD4i epitopes, as measured by an HIV-2 virus pre-triggered with sub-maximal concentrations of soluble CD4 (Decker et al., 2005), were previously reported to be present at approximately 5 wpi in only 5 of 14 (36%) individuals, and then, at titers that ranged from <20 to 823 (reciprocal IC_{50} serum dilution). At 5 months post infection, CD4i titers in these subjects had increased to a median reciprocal IC_{50} neutralizing titer of 38 (range, <20 to 631) and were detected in only 8 of 14 (57%) patients (Gray et al., 2007). Antibodies reactive with the HIV-1 MPER were found in even fewer subjects, with only 3 of 14 individuals exhibiting IC_{50} titers of between 28 and 51 during this study period (Gray et al., 2007). Additionally, the kinetics of appearance of autologous Nabs was delayed compared with the V3-specific antibody response. Upon enrollment into the CAPRISA cohort, none of the acutely infected subjects had detectable Nab titers against autologous enrollment virus (Table 1) and subsequent autologous Nab responses reached a median titer of only 261 by 5 months post infection (Fig. 2B and Table 1). A comparison with V3-specific antibodies, which reached median titers of >8000 by 5 months post-infection and were present in all individuals, demonstrates that antibodies targeting the V3 loop developed first and to higher titers compared with antibodies of CD4i, MPER, or strain-specific (autologous) specificities. The discordance between the kinetics of appearance and titers of V3 Nabs versus autologous strain-specific Nabs, and the breadth of reactivity displayed by V3 Nabs versus autologous antibodies, demonstrate that V3-specific antibodies contribute little if anything to the neutralization of autologous virus during acute, early, or chronic clade C HIV-1 infection. Our findings thus corroborate observations published by Moore et al. (2008) in which the autologous Nab responses were mapped to epitopes distinct from the HIV-1 V3.

Our analyses of two patients acutely infected with clade B HIV-1 indicated that broadly reactive V3-specific antibodies are elicited to high titers during acute clade B infection (Fig. 4 and Table 2). The data for the clade B HIV-1 infected subjects support our conclusions that V3-specific antibodies do not contribute to autologous or heterologous neutralization. For example, plasma drawn from patient BORI on day 218 post onset of symptoms contained V3-specific antibodies that neutralized the HIV-$2_{KR.X7}$YU2 V3 chimera at a reciprocal IC_{50} titer of >11,000 (Table 2) but this same plasma failed to neutralize the primary HIV-1_{YU2} strain at a 1:10 dilution (Wei et al., 2003). Additionally, HIV-$2_{KR.X7}$HIV-1 V3 chimeras containing HIV-1_{BORI} V3 sequences from day 6 and day 556 post onset of symptoms were constructed (Fig. 1B) and tested against the longitudinal BORI plasmas. We again detected high titer V3-specific reactivity in day 218 plasma (reciprocal IC_{50} plasma dilutions of 9038 and 6868 for HIV-$2_{KR.X7}$BORI$_{d6}$ and HIV-$2_{KR.X7}$BORI$_{d556}$ chimeras, respectively; Fig. 4 and Table 2). This potent V3-specific reactivity was not reflected in the autologous neutralization titers when tested against primary HIV-1_{BORI} virus (Wei et al., 2003). In addition, previous studies from our laboratory have shown that the primary HIV-1_{BORI} Env is highly resistant to two broadly reactive V3 mAbs, 447–52D and F425 B4e8, and to V3-specific antibodies in plasmas from chronically infected patients, while the HIV-$2_{KR.X7}$BORI$_{d6}$ V3 chimera, which presents the identical V3 loop, shows remarkable sensitivity to these mAbs and polyclonal plasma antibodies (Davis et al., 2009; Keele et al., 2008).

The finding that a broadly reactive high titer V3-specific antibody response is characteristic of acute HIV-1 infection prompted us to evaluate the specificities of the humoral response to clade B HIV-1 vaccine immunogens. Fig. 5D demonstrates that subjects receiving the ALVAC-HIV vCP1452 immunogen developed potent V3-specific Nabs against the HIV-$2_{KR.X7}$MN V3 chimera, which contains the V3

sequence identical to that included in the vaccine preparation, and that these V3-specific responses were moderately cross reactive at heterologous V3 epitopes as measured by the HIV-$2_{KR.X7}$YU2 V3 (clade B) and HIV-$2_{KR.X7}$Ccon V3 (clade C) chimeras. Addition of the AIDSVAX B/B monomeric gp120 protein boost to the immunization protocol resulted in equivalent neutralization titers against HIV-$2_{KR.X7}$MN V3, but substantially broadened neutralization against the chimeras containing heterologous clade-matched and cross-clade V3 epitopes. Median reciprocal IC_{50} neutralizing titers for these sera were 1527 against HIV-$2_{KR.X7}$MN V3, 3137 against HIV-$2_{KR.X7}$YU2 V3, and 1997 against HIV-$2_{KR.X7}$Ccon V3. In a previous study, we determined CD4i Nab titers in these same vaccines (Decker et al., 2005). We found that 3 of 5 individuals receiving the ALVAC-HIV vCP1452 based immunogen had detectable CD4i Nabs at a median titer of 23 (range, <20 to 53). All five subjects receiving the ALVAC-HIV vCP1452/AIDSVAX vaccine formulation developed detectable CD4i antibodies at a median reciprocal IC_{50} neutralizing titer of 179 (range, 53 to 357), significantly lower than the V3-specific titers measured for these individuals. Thus, we could conclude in this limited number of vaccinees that V3 was more immunogenic than CD4i epitopes, similar to our findings in individuals with acute and early HIV-1 infection.

Analysis of neutralizing activity against autologous and heterologous HIV-1 strains by vaccinee sera also demonstrated similarities to the antibody responses elicited during natural HIV-1 infection. Sera obtained from subjects receiving both the ALVAC-HIV vCP1452 and ALVAC vCP1452/AIDSVAX vaccine formulations exhibited potent neutralization against HIV-$2_{KR.X7}$MN V3, reaching titers >1000. These same immune sera, however, neutralized the autologous HIV-1_{MN} isolate at titers at least 10-fold lower, demonstrating that the neutralizing potency of V3-specific antibodies is significantly reduced when the V3 is presented in its native HIV-1 Env. This result was corroborated by an analysis of a second virus targeted by sera from subjects who received the ALVAC-HIV vCP1452/AIDSVAX immunogen. Sera from these vaccinees contained Nabs against the HIV-$2_{KR.X7}$YU2 V3 chimera with a median reciprocal IC_{50} neutralizing titer of >3000, but they were ineffective at neutralizing the primary HIV-1_{YU2} virus containing an identical V3 sequence at titers of 1:20. These findings demonstrate that while these HIV-1 Env immunogens readily elicited V3 specific antibodies with broad neutralizing potential, the potency of these antibodies against primary viruses is low.

Together, these data highlight important similarities in the V3-specific antibody responses elicited during acute and chronic HIV-1 infection and following vaccination with HIV-1 immunogens. In all cases, V3-specific antibodies were elicited early and to high titers. These V3 reactive antibodies were of substantial breadth, capable of mediating neutralization against within-clade and cross-clade V3 epitopes. However, these antibodies exhibited reduced neutralizing potency against autologous HIV-1 isolates and failed to neutralize typical primary HIV-1 strains at all. These observations highlight the exceptional V3 shielding that is characteristic of primary HIV-1 viruses (Davis et al., 2009; Krachmarov et al., 2005, 2006; Pinter, 2007), which effectively limits the contributions of V3-specific antibodies to virus neutralization in acute and chronic HIV-1 infection and subsequent to vaccination with HIV-1 immunogens. These data contribute to a growing body of evidence showing that while HIV-1 V3 is highly immunogenic and elicits antibodies that recognize V3 epitopes that are conserved among diverse HIV-1 strains (Davis et al., 2009; Goudsmit et al., 1988; Krachmarov et al., 2005; Wu et al., 2006; Zolla-Pazner et al., 2008), such antibodies are generally poor effectors of neutralization of primary viruses due to concealment of V3 epitopes on the native HIV-1 Env trimer (Davis et al., 2009; Krachmarov et al., 2005, 2006). If V3 directed antibodies are to be a component of an effective HIV-1 vaccine, such shielding of V3 must be overcome.

Materials and methods

Study subjects

Fourteen women with acute HIV-1 clade C infection were studied. These individuals were part of the CAPRISA cohort (Gray et al., 2007; Moore et al., 2008) of high risk sex workers in Durban, South Africa, who had been counseled regarding safe sex practices and followed prospectively by monthly testing for plasma HIV-1 RNA and antibodies for incident HIV-1 infection. The timing of HIV-1 infection was estimated by CAPRISA investigators to be the midpoint between the last HIV-1 antibody negative sample and the first antibody positive sample or 14 days before the individual was found to be plasma HIV-1 vRNA positive and antibody negative. This method for estimating the date of virus infection may underestimate the actual duration between virus transmission and sampling in a substantial fraction of newly infected individuals and thus comparisons of the timing of antibody responses between different studies must be done with this caveat in mind. Two subjects with acute HIV-1 clade B infection were identified based on symptomatic primary infection (Wei et al., 2003) at the University of Alabama at Birmingham. Following the diagnosis of acute HIV-1 infection, all 16 subjects had serial phlebotomies on a weekly, monthly, and quarterly basis for the collection and cryopreservation of serum, plasma and peripheral blood mononuclear cells. None of the HIV-1 infected subjects received antiretroviral therapy during the course of follow-up described in this study. Samples from fifteen HIV-1 uninfected study participants in HVTN Protocol 203 study were obtained from the NIAID HIV Vaccine Trials Network (http://www.hvtn.org/). All study protocols received Institutional Review Board approval and study subjects provided informed consent.

Molecular cloning and mutagenesis

The construction of pHIV-$2_{KR.X4}$, pHIV-$2_{KR.X7}$, and pHIV-$2_{KR.X7}$HIV-1 V3 chimeras containing V3 sequences from HIV-1_{MN} (Accession number: M17449, Gurgo et al., 1988), HIV-1_{YU2} (Accession number: M93258, Li et al., 1992) or HIV-1_{Ccon} (Los Alamos Sequence Database: http://www.hiv.lanl.gov/) has been described (Davis et al., 2009). Substitution of V3 sequences from HIV-1_{WEAU} (Accession number: AY223750, Wei et al., 2003), HIV-1_{BORI_d6} (Accession number: AY223720, Keele et al., 2008; Wei et al., 2003) and HIV-1_{BORI_d556} (Accession number: AY223732, Wei et al., 2003) was completed using a modified version of the QuikChange™ Site-Directed Mutagenesis protocol (Stratagene, La Jolla, CA). 5′ phosphorylated (Phos) primers were designed to anneal to the HIV-2_{KR} backbone immediately flanking the HIV-$2_{KR.X7}$ V3 region and contained the desired HIV-1 V3 substitutions as follows: HIV-1_{WEAU} V3: GTACTTTATACAACAGGAGAAATAATAGGAGATATAAGACGAGCACATTGCTGGTTCGGAGGTGATTGG, [Phos] TCTCCCTGGTCCTAAAGTTATCCCTTTTCTTGTATTGTTGTTGGGTCTTATACAATGCATTGTGAG; HIV-1_{BORI_d6}: ACATTTTATACAACAGGAGACATAATAGGAGATATAAGGCAGGCATATTGCTGGTTCGGAGGTGATTGG, [Phos] TCTCCCTGGTCCTATATGTATACTTTTTCTTGTATTGTTGTTGGGTCTTGTACAATGCATTGTGAG; HIV-1_{BORI_d556}: ACATTTTATACAACAGGAGACATAATAGGAAATATAAGGCAAGCACATTGCTGGTTCGGAGGTGATTGG, [Phos] TCTCCCTGGTCCTATATGTATACCTTTTCTTGTATTGTTGTTGGGTCTTGTACAATGCATTGTGAG (Operon Biotechnologies, Inc., Huntsville, AL). Fifteen pmol of each primer and 100 ng of template DNA [pGEM subclone containing a 1.2 kb Env fragment of pHIV-$2_{KR.X7}$MN V3(Davis et al., 2009)] were used for each PCR mutagenesis reaction. PCR conditions were as follows: 95°C for 1 min (1 cycle); 95°C for 50 s, 50°C for 50 s, 68°C for 6 min (18 cycles); 68°C for 7 min (1 cycle). PCR products were digested with 10 U of DpnI (New England Biolabs; Ipswich, MA) at 37°C for 1 h to cleave template DNA and heat inactivated at 72°C for 30 min. Amplification products were ligated to yield a chimeric *env*

construct with appropriate V3 substitutions and subsequently cloned into the full-length pHIV-$2_{KR.X7}$ backbone using the *XhoI* and *XmaI* restriction sequences. Plasmid DNA was transformed into XL2-Blue MRF' Ultracompetent cells (Stratagene, La Jolla, CA), plated on LB agar plates supplemented with 5 μg/ml kanamycin and cultured overnight at 30°C. Single colonies were selected and grown overnight in liquid LB broth containing 5 μg/ml kanamycin at 30°C with 225 rpm shaking followed by plasmid isolation. Each molecular clone was confirmed by nucleotide sequencing.

Virus stocks

3×10^6 293T cells were seeded into 10 cm^2 tissue culture dishes and cultured overnight in complete Dulbecco's modified Eagle medium (DMEM; Gibco/Invitrogen, Carlsbad, CA) supplemented with 10% FBS and 1% PS (D10 media) at 37°C with 5% CO_2. The following day, 6 μg proviral DNA was transfected into 293T cells using Fugene-6 (Roche Applied Science, Indianapolis, IN) as specified by the manufacturer and cultured in D10 media at 37°C for 48 h. Supernatants containing infectious virus were harvested, cleared of cellular debris by low-speed centrifugation, and used directly or stored at −80°C for future use.

Virus titration

1×10^4 TZM-bl reporter cells per well were seeded in D10 media and cultured in 96-well tissue culture plates (BD Falcon, Franklin Lakes, NJ) overnight at 37°C with 5% CO_2. The following day, D10 media was removed from the cell monolayers and replaced with 50 μl DMEM, 6% FBS, 1% PS (D6 media) containing DEAE-Dextran hydrochloride at 80 μg/ml (2× final concentration). Serial five-fold dilutions of virus stock were prepared in D10 media. Fifty μl virus stock dilution was then transferred to the TZM-bl reporter cells (catalog #8129; NIH AIDS Research and Reference Reagent Program) bringing the final volume to 100 μl and final DEAE-Dextran concentration to 40 μg/ml. Cells were incubated with virus for 48 h at 37°C, lysed, and analyzed for luciferase production using the Luciferase Assay System (Promega, Madison, WI). Future neutralization experiments were performed using approximately 1×10^5 RLU/well (96-well format) of virus stock.

Neutralization assays

For these studies, 1×10^4 TZM-bl cells per well were seeded into 96-well tissue culture plates and cultured overnight in D10 media. Six five-fold serial serum dilutions were prepared in D6 media. Virus 293T stock supernatants were prepared as described previously and diluted to approximately 1×10^5 RLU/well in D10 media containing 80 μg/ml DEAE-Dextran hydrochloride. Equal parts serum and virus dilutions were then combined and incubated at 37°C for 1 h. Media was removed from the TZM-bl cells, 100 μl of the virus/serum mixture was applied to the cells and incubated for an additional 48 h at 37°C with 5% CO_2. Control wells included a virus-only control (no serum) and a media-only control (no serum, no virus). Luciferase expression was quantified according to the Luciferase Assay System protocol (Promega, Madison, WI). All wells except the starting serum dilution were supplemented with 2% (for a 1:50 starting dilution) or 10% (for a 1:10 starting dilution) normal human serum.

Peptide and fusion protein competition assays

Linear peptides comprising full-length V3 sequences (33-mer) from autologous CAPRISA viruses were purchased from NMI peptides (Reutlingen, Germany) and supplied by the NICD (Moore et al., 2008). Inhibition experiments were performed as described above for serum neutralization assays, except that serum dilutions were preincubated with peptide (final concentration, 50 μg/ml) for 30 min at 37°C prior to incubation with virus. Fusion protein (Fc-$V3_C$ FP) consisted of a rabbit IgG Fc molecule with a complete HIV-1_{Ccon} V3 loop linked to its N-terminus (A.P. and A.S., unpublished, and Davis et al., 2009). Fusion protein inhibition studies were performed as described above for the peptide inhibition assays with Fc-$V3_C$ FP at a final concentration of 10 μg/ml. Peptide and Fc-V3 FP concentrations were kept constant in all wells including control wells. Controls included scrambled V3 peptide and rabbit IgG Fc alone or rabbit IgG Fc-V3 containing a Gly-Ala-Gly linker to replace the 19 amino acids of the V3 tip residues [Fc-$V3_{p8027}$ (Davis et al., 2009)].

Statistical analyses

Median reciprocal IC_{50} neutralization titers were compared by the Wilcoxon Rank Sum test using the SAS version 9.1 software package. A *p*-value of 0.05 was considered significant for these studies.

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