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Changing sensitivity to broadly neutralizing antibodies b12, 2G12, 2F5, and 4E10 of primary subtype B human immunodeficiency virus type 1 variants in the natural course of infection

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ABSTRACT

The conserved nature of the epitopes of the four broadly neutralizing antibodies (BNAbs), b12, 2G12, 2F5, and 4E10, may imply that the sensitivity of HIV-1 for these BNAbs remains fairly constant over the course of infection. Here, we demonstrate that viruses isolated early during the course of infection were mostly sensitive to HIVIg and antibody neutralization, although variation was observed in neutralization sensitivity of coexisting viruses to the different antibodies as well as between viruses from different patients. HIV-1 resistance to HIVIg developed relatively early during follow-up in three out of five patients, while early, b12 sensitive viruses in three out of five patients were replaced by b12 resistant variants relatively late in infection. In contrast, viruses generally remained sensitive to 2F5 and 4E10 neutralization over the course of infection, although 2F5 and/or 4E10 resistant variants did emerge later in infection in four out of five patients. In most patients, HIV-1 resistance to 2F5 or 4E10 did not correlate with mutations at critical amino acid positions in their defined epitopes. Viruses resistant to 2G12-mediated neutralization were present throughout the course of infection. As viral resistance against BNAb-mediated neutralization generally developed when autologous serum neutralizing activity had faded, it seems unlikely that these changes are driven by escape from autologous humoral immunity.

Introduction

The humoral immune response which develops in the natural course of human immunodeficiency virus type 1 (HIV-1) infection generally has limited cross-reactivity, although a subset of individuals may develop broadly neutralizing activity in serum later in infection (Binley et al., 2008; Dhillon et al., 2007; Li et al., 2009; Sather et al., 2009; Shen et al., 2009). It is still unclear if broadly neutralizing activity in serum is the resultant of a single highly effective antibody, or of different antibody specificities which in combination give a cross-reactive neutralizing phenotype. Although a recent study points towards the latter possibility (Scheid et al., 2009), a small number of broadly neutralizing antibodies (BNAbs) have been isolated from HIV-infected individuals, of which the four best characterized are b12, 2G12, 2F5, and 4E10 (Burton et al., 1994; Muster et al., 1993; Stiegler et al., 2001; Trkola et al., 1995).

Antibody b12 recognizes a conformational epitope which partially overlaps the CD4 binding site in gp120 (Saphire et al., 2001), whereas 2G12 is directed against a cluster of carbohydrate residues on the surface of gp120 close to the coreceptor binding domain (Sanders et al., 2002; Scanlan et al., 2002). 2F5 and 4E10 bind to adjacent linear epitopes located within the membrane-proximal external region (MPER) of gp41 (Cardoso et al., 2005; Ofek et al., 2004; Zwick et al., 2005). The fact that these epitopes are conserved on the viral envelope may explain the broadly neutralizing phenotype of the antibodies by which they are targeted. However, even for these BNAbs some subtype-specific neutralization patterns have been observed (Binley et al., 2004). Indeed, the neutralizing potency of BNAbs b12 and 2G12 is higher against subtype B HIV-1 than to HIV-1 variants from other subtypes (Binley et al., 2004), which may relate to the fact that these antibodies were isolated from subtype B HIV-1 infected individuals. The MPER is more conserved between different subtypes, explaining the relatively large neutralization breadth of the anti-gp41 Abs, albeit that the potency of the 4E10 antibody is lower than that of the other BNAbs (Binley et al., 2004). Although the MPER is relatively conserved, the 2F5 epitope is absent in a large proportion of subtype C viruses, which explains the limited neutralizing activity of the 2F5 antibody against HIV-1 variants of this subtype (Binley et al., 2004; Li et al., 2006).

We and others have previously analyzed primary HIV-1 from acute, early and chronic stages of infection for their sensitivities to BNAb

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neutralization, and could correlate neutralization resistance to a certain BNAb with mutations in the corresponding epitope (Binley et al., 2004; Li et al., 2005, 2006; Quakkelaar et al., 2007; Richman et al., 2003; Rusert et al., 2005). Interestingly, neutralization resistance was also observed in the absence of mutations in the epitope (Binley et al., 2004; Quakkelaar et al., 2007), indicating that mutations outside the epitope may influence the structural context of the envelope and thereby the exposure of the epitopes for these BNAbs. Since b12, 2G12, 2F5, and 4E10 target conserved regions of the viral envelope, and considering the fact that these antibody specificities are rare, which will limit the selection pressure on these regions, one might expect that the epitopes of these BNAbs are well preserved, and that the sensitivities of virus variants throughout the clinical course of infection for these BNAbs will remain relatively constant. To study the extent of change in BNAb susceptibility of HIV-1 within an individual over the course of infection, we performed a longitudinal analysis of the sensitivities to BNAbs b12, 2G12, 2F5, and 4E10 of primary HIV-1 variants that were isolated from five typical progressors during the course of HIV-1 infection. In addition, we analyzed the envelope sequences for variation in the antibody epitopes that correlated with sensitivity of the viruses for the respective antibodies.

Results

Patients and viruses

We investigated the in vitro sensitivity for HIVIg, a pooled IgG from HIV$^+$ individuals, and 4 broadly neutralizing antibodies (BNAbs), of subtype B HIV-1 variants that were isolated at multiple time points over the course of infection from five participants of the ACS. From these individuals, who progressed to AIDS in 7–11 years and who did not develop X4 HIV-1 variants, we have previously isolated clonal HIV-1 variants from PBMCs that were obtained at six time points covering the disease course from SC up to 2 to 3 years after clinical AIDS diagnosis (Bunnik et al., 2008). A minimum of two and a maximum of five clonal variants per time point were analyzed for their neutralization sensitivity. For patient H5, attempts to isolate clonal HIV-1 variants from PBMCs obtained at SC and at time points after AIDS diagnosis were not successful. In a previous study, we determined that individuals H1, H2, and H3 developed high-titer autologous neutralizing activity, which decreased in potency during chronic infection, whereas autologous neutralizing activity was not detected in serum from patients H4 and H5 (Bunnik et al., 2008). Neutralization sensitivity for the broadly neutralizing monoclonal antibodies was assessed in a PBMC-based assay, using p24 production as a read-out for virus replication. As HIVIg contains anti-p24 antibodies which interfere with the detection of p24, we used a TZM-bl-based assay for measuring sensitivity to neutralization by HIVIg.

Sensitivity of longitudinally isolated primary HIV-1 to HIVIg neutralization

The majority of viruses from all timepoints and from all patients were resistant to neutralization by HIVIg, even at the highest concentration tested (500 μg/ml; Fig. 1). Virus variants from the

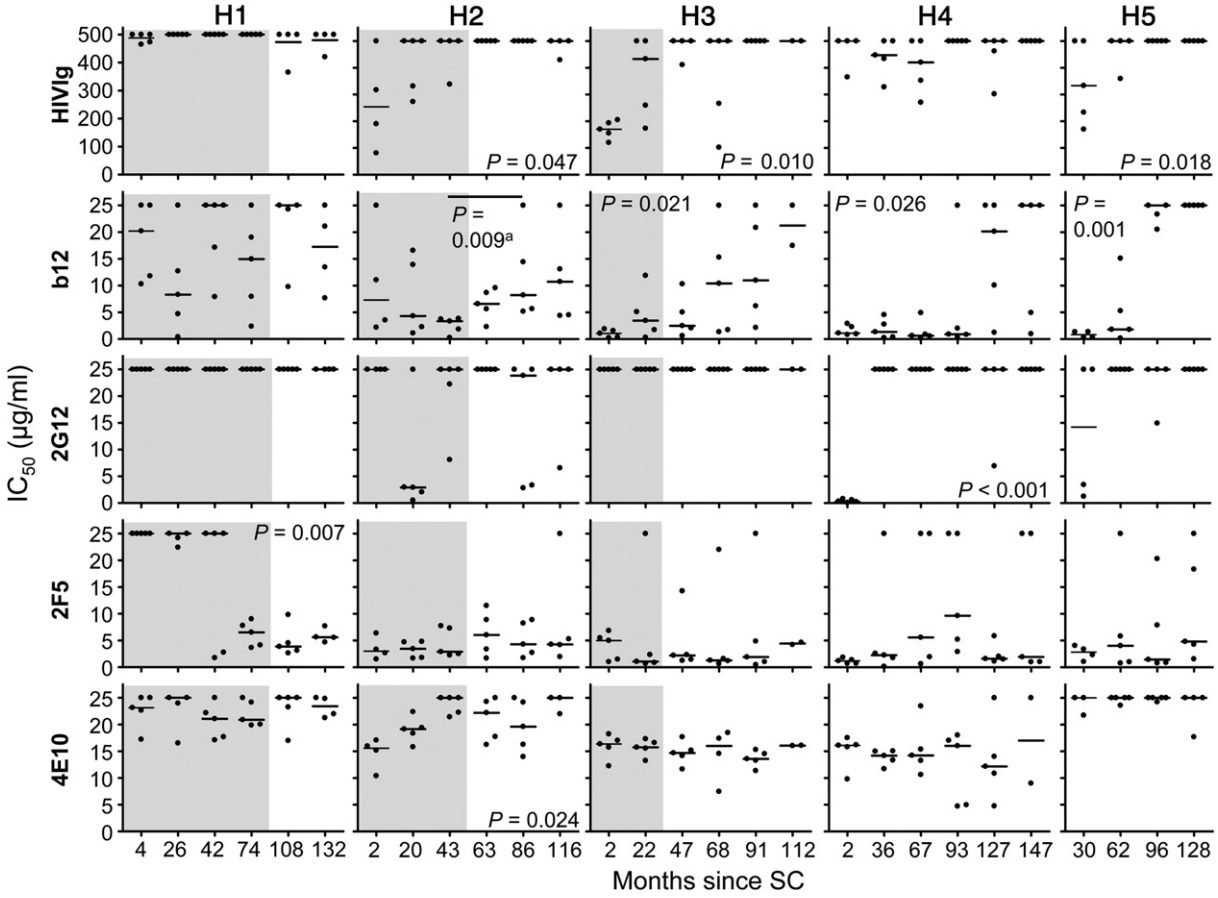

Fig. 1. Sensitivity for neutralization by HIVIg, b12, 2G12, 2F5, and 4E10 of longitudinally obtained virus variants from five typical progressors. IC_{50}s of individual virus clones as determined by linear regression are shown. The horizontal lines represent the median IC_{50}s. Shaded areas indicate the time points where autologous neutralizing activity was detected (Bunnik et al., 2008). The fifth time point in patients H1 to H4 is close to the moment of clinical AIDS diagnosis; for patient H5 the fourth time point is closest to AIDS diagnosis. Longitudinal changes in neutralization sensitivity were assessed using a Kruskal–Wallis test. [a] Mann–Whitney *U* test.

earliest time points from patients H2, H3, and H5 were more sensitive to HIVIg neutralization than viruses from the later time points ($P=0.047$, $P=0.010$ and $P=0.018$, respectively), indicating that the virus populations in these patients became more resistant to antibody neutralization during the course of infection. In patients H2 and H3, changes in sensitivity for HIVIg coincided with the presence of autologous neutralizing activity in serum. The increasing resistance against HIVIg mediated neutralization of viruses from patient H5 also occurred during the asymptomatic period, but in the absence of detectable autologous neutralizing activity. Early virus variants of patients H1 and H4 were already relatively resistant to HIVIg neutralization and no significant alterations in sensitivity to HIVIg neutralization were observed over time in these individuals.

Sensitivity of longitudinally isolated primary HIV-1 to b12 neutralization

Clonal HIV-1 variants that were isolated shortly after SC from patients H3, H4, and H5 were highly sensitive to neutralization by b12 (IC_{50} values of individual viruses varied between 0.33 and 2.91 μg/ml; Fig. 1). In these three patients, the median IC_{50} per time point for b12 increased during the course of infection ($P=0.021$, $P=0.026$, and $P=0.001$, respectively). Moreover, at least 50% of the virus variants that were isolated at the latest time point from PBMC from each of these three individual were resistant to b12 neutralization, even at the highest concentration tested (25 μg/ml). In patient H2, a large variation in the sensitivity for b12 neutralization was observed among the earliest virus variants, ranging from 50% neutralization at 2.18 μg/ml to resistance against neutralization at 25 μg/ml b12. In contrast, viruses isolated at the midpoint of the asymptomatic phase of infection were with no exception highly sensitive to b12 neutralization. Resistance to b12 mediated neutralization increased again late in infection, as the late virus variants had significantly higher IC_{50}s compared to the b12 sensitive virus variants isolated during asymptomatic infection ($P=0.009$). Interestingly, in these four patients, the major changes in sensitivity for b12 neutralization occurred late in infection, when autologous neutralizing activity was no longer present. In patient H1, changes in sensitivity to b12 neutralization of clonal HIV-1 variants isolated during the course of infection were not observed. At all time points, most viruses isolated from patient H1 were moderately sensitive to b12 neutralization, although coexisting HIV-1 variants that resisted b12-mediated neutralization were also present.

Sensitivity of longitudinally isolated primary HIV-1 to 2G12 neutralization

With the exception of the earliest viruses from patient H4 and a minority of viruses isolated from patients H2 and H3, all virus variants analyzed here were resistant to neutralization by 2G12 (Fig. 1). Resistance to 2G12 neutralization correlated well with an envelope sequence that predicted the absence of one or more N-linked glycans that together form the 2G12 epitope (Sanders et al., 2002; Scanlan et al., 2002) (Table 1). HIV-1 variants from patients H1 and H5 lacked an N at position 339 (N339X) at all time points that were analyzed in the course of infection. In patient H4, resistance to 2G12 neutralization of virus variants isolated at later time points in the course of infection coincided with the loss of either N386 or N392, whereas the absence of N295, N332 and/or N339 was observed in 2G12 resistant HIV-1 variants from patient H3. 2G12 neutralization sensitive and resistant viruses coexisted in patient H2 throughout the course of infection. 2G12 resistant variants in this patient missed one or more PNGS at positions 295, 339, 386 and/or 339.

Sensitivity of longitudinally isolated primary HIV-1 to 2F5 neutralization

The earliest virus variants isolated from patients H2, H3, H4, and H5 were all highly sensitive to 2F5 neutralization (Fig. 1). Over the course of infection, the majority of virus variants remained sensitive to 2F5 neutralization (Fig. 1), although at one or more time points during infection, each of these four patients harboured a minority of viruses that resisted 2F5 neutralization even at 25 μg/ml. In viruses from patients H2, H4, and H5, sensitivity to 2F5 neutralization correlated well with the absence of mutations in the 2F5 epitope (data not shown). 2F5 neutralization sensitive viruses from patient H3 did contain a number of mutations in the core 2F5 epitope, but these did not involve the central DKW sequence (Zwick et al., 2005) (Table 2). In agreement with previous observations, mutations in the 2F5 epitope were also absent in a small number of viruses that completely resisted 2F5 neutralization (Binley et al., 2004; Quakkelaar et al., 2007), which may indicate that the 2F5 epitope is not equally exposed on all viruses.

Virus variants isolated early in infection from patient H1 contained a glutamine residue at position 665 in the 2F5 epitope (DQW instead

Table 1
Average 2G12 IC_{50} for HIV-1 variants with similar 2G12 epitope per time point and presence or absence of PNGS of the 2G12 epitope for corresponding viruses.

Patient	Mo since SC	*n*	2G12 epitope[a]					Avg IC_{50} (μg/ml)
			295	332	339	386	392	
H1	4	5/5	+	−	−	+	+	>25
	26	3/5	−	+	−	+	+	>25
		1/5	−	+	−	−	+	>25
		1/5	−	−	−	+	+	>25
	42	3/5	−	+	−	+	−	>25
		2/5	−	+	−	+	+	>25
	74	5/5	−	+	−	+	−	>25
	108	3/5	−	+	−	−	−	>25
		2/5	−	+	−	+	−	>25
	132	2/4	−	+	−	+	−	>25
		2/4	−	+	−	−	−	>25
H2	2	4/4	+	+	−	+	+	>25
	20	4/5	+	+	+	+	+	2.1
		1/5	+	+	+	−	+	>25
	43	3/5	+	+	+	−	+	>25
		2/5	+	+	+	+	+	18.4
	63	2/5	+	+	+	+	−	>25
		1/5	+	+	+	+	+	>25
		1/5	+	+	+	−	+	>25
		1/5	+	+	−	+	−	>25
	86	2/5	+	+	+	+	+	3.1
		2/5	+	+	+	−	+	>25
		1/5	−	+	+	+	+	23.9
	116	2/5	+	+	+	+	+	15.8
		2/5	+	+	−	−	−	>25
		1/5	+	+	+	−	+	>25
H3	2	5/5	−	+	+	+	+	>25
	22	4/5	−	−	+	+	+	>25
		1/5	+	−	+	+	+	>25
	47	5/5	+	−	+	+	+	>25
	68	2/5	+	−	+	+	+	>25
		2/5	+	−	−	+	+	>25
		1/5	−	−	+	+	+	>25
	91	4/5	+	−	−	+	+	>25
		1/5	−	−	+	+	+	>25
	112	2/2	+	−	−	+	+	>25
H4	2	5/5	+	+	+	+	+	0.4
	36	3/5	+	+	+	−	+	>25
		2/5	+	+	+	+	−	>25
	67	4/5	+	+	+	−	+	>25
	93	3/5	+	+	+	+	−	>25
		2/5	+	+	+	−	+	>25
	127	4/5	+	+	+	+	−	>25
		1/5	+	+	+	+	+	6.93
	147	5/5	+	+	+	+	−	>25
H5	30	5/5	+	+	−	+	+	15.9
	62	4/5	+	+	−	+	+	>25
		1/5	+	+	−	−	+	>25
	96	5/5	+	+	−	+	+	23.0
	128	5/5	+	+	−	+	+	>25

Mo, months; SC, seroconversion; Avg, average; *n*, number of viruses/total number of viruses tested.
[a] +, PNGS present; −, PNGS absent.

Table 2
Average 2F5 IC_{50} per time point and 2F5 epitope sequence variant of primary HIV-1 isolated at sequential time points from patients H1 and H3.

Patient	Mo since SC	*n*	2F5 epitope ELDKWAS[a] (aa 662–668)	Avg IC_{50} (μg/ml)
H1	4	5/5	...Q...	>25
	26	5/5	...Q..N	24.3
	42	3/5	...Q...	>25
		2/5	......N	2.3
	74	5/5	.....	6.2
	108	5/5	.....	4.8
	132	4/4	.....	5.9
H3	2	5/5	......N	2.5
	22	5/5	......N	6.0
	44	3/5	.....SN	1.7
		2/5	.....TN	8.2
	68	3/5	.....SN	8.0
		1/5	.W...SN	1.6
		1/5	A....SN	1.3
	91	3/5	A....SN	2.6
		2/5	.....SN	12.7
	119	1/2	.....SN	4.2
		1/2	A....SN	4.7

Mo, months; SC, seroconversion; aa, amino acid; Avg, average; *n*, number of viruses/total number of viruses tested.
[a] Essential amino acid residues in the core 2F5 epitope are indicated in bold (Zwick et al., 2005).

of DKW), which rendered these viruses resistant to neutralization by 2F5 (Table 2). During asymptomatic infection, this mutation reverted to a lysine which coincided with an increased sensitivity for 2F5 (Table 2). The earliest virus population in patient H1 already contained a glutamine residue at position 665, indicating that the K665Q substitution was most likely present in the transmitted virus variant. Unfortunately, since the donor of patient H1 is unknown to us, we were not able to study whether this mutation may have been introduced under the selective pressure of 2F5-like antibodies in the previous host. However, we determined that sera from patient H1 from several time points during infection did not contain neutralizing activity against an HIV-2 variant engrafted with the 2F5 epitope from HIV-1 (a kind gift from G. Shaw; data not shown). These results indicate that patient H1 did not develop 2F5-like antibodies at any time during infection, suggesting that the absence of 2F5-like antibodies may have allowed the reversion of the K665Q mutation.

Sensitivity of longitudinally isolated primary HIV-1 to 4E10 neutralization

Virus variants from patients H2, H3, and H4 were moderately sensitive to 4E10 neutralization, while virus variants with higher levels of resistance were present at all time points in patients H1 and H5 (Fig. 1). Increasing numbers of viruses resistant to 4E10 even at 25 μg/ml were observed during the asymptomatic and symptomatic phase of infection in patients H2 and H4, which in patient H2 coincided with the presence of autologous neutralizing activity in serum.

In all patients, HIV-1 variants with substitutions in the 4E10 epitope were observed throughout infection (Table 3), although none of the mutations was located at positions critical for 4E10 recognition (Zwick et al., 2001). However, the substitutions at position 677 in the viruses from patient H5 might play a role in their relative resistance to 4E10 neutralization.

Resistance against BNAbs of longitudinally isolated primary HIV-1

To better understand the extent of resistance against BNAbs over the course of infection, we used the neutralization data as presented in Fig. 1 to determine the percentage of neutralization resistant virus variants per time point for HIVIg (IC_{50} >500 μg/ml) and each of the four BNAbs (IC_{50} >25 μg/ml), as well as the percentage of virus variants per time point that resisted neutralization by one or more BNAbs (Fig. 2). Overall, most virus variants analyzed in this study were resistant against one or more BNAbs, which in most cases included 2G12. As can be expected from the distinct localization of the antibody epitopes on the viral envelope, resistance to one BNAb was not predictive for sensitivity to another BNAb (data not shown).

Only at the earliest time point in patient H4, all virus variants tested sensitive to neutralization by all four BNAbs, although even most of these viruses were resistant to HIVIg. In patients H4 and H5,

Table 3
4E10 epitope sequence of primary HIV-1 variants and average 4E10 IC_{50} for HIV-1 variants with similar 4E10 epitope sequence per time point from typical progressors H1, H2, H3, H4, and H5.

Patient	Mo since SC	*n*	Epitope 4E10 NWFDITNWLW[a] (aa 671–680)	Avg IC_{50} (μg/ml)
H1	4	5/5	...S.....	22.6
	26	5/5	.....	23.1
	42	3/5	......K...	21.0
		2/5	.....	19.9
	74	3/5	......K...	21.7
		2/5	.....SK...	22.4
	108	5/5	.....SK...	23.0
	132	4/4	.....SK...	23.3
H2	2	4/4	S.....	14.7
	20	2/5	......S...	17.6
		2/5	......K...	20.8
		1/5	.....	18.3
	43	2/5	.....SS...	>25
		1/5	......S...	21.4
		1/5	......S...	22.3
		1/5	.....P.	>25
	63	2/5	.....	19.2
		2/5	.....SK...	24.6
		1/5	.....SR...	17.7
	86	2/5	.....	21.9
		2/5	.....SK...	19.5
		1/5	.....SS...	16.3
	117	2/5	.....	n.d.
		1/5	...N.SS...	>25
		1/5	.....SS...	>25
		1/5	...S.SS...	22.0
H3	2	5/5	.....S....	15.9
	22	4/5	.....S....	16.3
		1/5	.-...S....	13.2
	44	2/5	...N.S....	15.9
		2/5	T....S....	13.2
		1/5	T.....	15.2
	68	3/5	T.....	13.1
		2/5	...N.S....	18.5
	91	4/5	T.....	13.2
		1/5	T....S....	15.3
	119	2/2	T.....	16.1
H4	2	5/5	.....S....	15.1
	36	4/5	.....SK...	13.6
		1/5	...N.S....	15.0
	67	4/5	.....S....	15.4
		1/5	.....SK...	15.4
	93	3/5	.....SK...	8.6
		2/5	.....S....	17.5
	127	4/5	.....SK...	13.7
		1/5	.....S....	12.2
	147	5/5	.....SK...	17.0
H5	30	3/5	......H...	>25
		2/5	......S...	23.4
	62	2/5	......H...	>25
		2/5	......S...	>25
		1/5	......R...	23.6
	96	4/5	......H...	>25
		1/5	......S...	24.2
	128	5/5	......H...	23.5

Mo, months; SC, seroconversion; aa, amino acid; Avg, average; *n*, number of viruses/total number of viruses tested.
[a] Amino acid residues essential for 4E10 recognition are indicated in bold, other contact residues are underlined (Cardoso et al., 2005; Zwick et al., 2005).

Patient	Mo since SC	% of HIV-1 variants resistant to HIVIg	b12	2F5	2G12	4E10	% of HIV-1 variants resistant to n BNAbs 0	1	2	3	4
H1	4	60	40	100	100	40			40	40	20
	26	100	20	60	100	60		20	20	60	
	42	100	60	60	100	20		20	40	20	20
	74	100	20		100	20		80		20	
	108	100	60		100	60		20	40	40	
	132	75	25		100	25		50	50		
H2	2	25	25		100			75	25		
	20	60			20		80	20			
	43	80			60	60	20	40	40		
	63	100			100	20		80	20		
	86	100	20		40	20	60		40		
	116	80	20	20	80	66,7		60	20	20	
H3	2	0			100			100			
	22	40		20	100			80	20		
	47	80			100			100			
	68	60	20		100			80	20		
	91	100	20	20	100			60	40		
	112	100	50		100			50	50		
H4	2	80					100				
	36	40		20	100			80	20		
	67	40		40	100			60	40		
	93	100	20	40	100			60	20	20	
	127	60	40		80	20	20	40	20	20	
	147	100	60	40	100	50		40		60	
H5	30	40			50	66,7	25	50	25		
	62	80		20	100	80		20	60	20	
	96	100	60		80	80		40		60	
	128	100	100	20	100	80			20	60	20

Fig. 2. Resistance to HIVIg, b12, 2G12, 2F5, and 4E10 of longitudinally obtained primary HIV-1. On the left, the percentage of virus variants per time point that resisted neutralization by HIVIg (at 500 μg/ml) and the four BNAbs (at 25 μg/ml) is shown. The right part shows the percentage of virus variants that resisted neutralization by a certain number of BNAbs at 25 μg/ml. The percentage of resistant viruses per time point is color coded using increasingly darker shades of grey. At grey-shaded time points, autologous neutralizing activity was previously detected (Bunnik et al., 2008). Mo, months.

who both lack autologous neutralizing activity in serum (Bunnik et al., 2008), the majority of the earliest virus variants were sensitive to three or four BNAbs. However, resistance for these BNAbs developed over the course of infection, accumulating at the latest time point in a majority of virus variants that resisted neutralization by at least 3 of the BNAbs. The majority of late virus variants from patient H3 resisted b12 neutralization, while resistance to the other BNAbs did not develop. In patient H1 the relative resistance to neutralization by all BNAbs persisted over time. In general, the absence or presence of strong autologous humoral immunity in a patient was not predictive for the emergence of virus variants that resisted neutralization by b12, 2G12, 2F5, or 4E10. Moreover, BNAb resistant viruses emerged in the absence of autologous neutralizing activity in serum.

Discussion

In the search for a vaccine that is capable of eliciting cross-reactive neutralizing antibodies, research has focused on the broadest neutralizing monoclonal antibodies that have been discovered to date: b12, 2G12, 2F5, and 4E10. Although other broadly neutralizing antibody (BNAb) specificities are likely to exist (Binley et al., 2008; Dhillon et al., 2007; Li et al., 2009), the viral epitopes of the four known BNAbs are currently the most interesting targets for vaccine design. For this reason, it is important to understand the degree of variation in susceptibility for these four BNAbs among circulating virus variants. Thus far, the BNAb sensitivities of smaller or larger panels of unrelated viruses have been comprehensively mapped (Binley et al., 2004; Li et al., 2005, 2006; Rusert et al., 2005). However, changes in neutralization sensitivity over the course of infection have mainly been studied in relation to the development of the autologous NAb response (Bunnik et al., 2008; Richman et al., 2003; Sagar et al., 2006; Wei et al., 2003), while it remains to be established whether viruses also evolve during the course of infection with regard to their sensitivity for BNAb.

Here, we performed a longitudinal analysis of the resistance against neutralization by b12, 2G12, 2F5, and 4E10 among primary HIV-1 from five typical progressors. In addition, we related changes in neutralization sensitivity to mutations in the respective antibody epitopes. In general, the earliest virus populations were most sensitive to BNAb neutralization, which might point towards the existence of a certain transmitted virus phenotype, which has previously been observed for subtype A and C HIV-1 in relation to neutralization sensitivity for sera of transmitting partners (Chohan et al., 2005; Derdeyn et al., 2004; Sagar et al., 2006). The fact that we determined sensitivity to broadly neutralizing antibodies may explain why we now confirm this observation for subtype B HIV-1 while this was not observed in previous studies in which sensitivity to neutralization by autologous and heterologous sera was tested (Chohan et al., 2005; Frost et al., 2005). However, HIV-1 infection can also be established by BNAb resistant viruses, as was observed in patient H1.

In patients who early in infection had viruses that were sensitive to BNAb neutralization, a gradual increase in resistance to neutralization by one or more BNAb was observed during later stages of disease. Most notably, virus populations changed from highly sensitive to b12 neutralization early in infection to partially or completely resistant to b12 neutralization in the late asymptomatic phase in three out of five patients. Previously published reports did not show this change in b12 neutralization sensitivity during infection (Keele et al., 2008; Rusert

et al., 2005), which might be explained by the fact that early and late viruses in these studies were not obtained from the same individuals, by differences in sampling dates of earliest viruses (between 2 and 4 months after estimated SC date in our study as compared to around the moment of seroconversion in the study by Keele et al.) or by our inclusion of viruses from extremely late time points during infection (112–147 months post-SC) while late viruses in the other studies were obtained from chronically infected patients at >24 months after SC. While our data are discrepant from previous observations for sensitivity to b12 neutralization, the observation that viruses from chronically infected patients in the study by Rusert et al. were more resistant to neutralization by 2F5 and 4E10 corresponds to the increasing numbers of (moderately) neutralization resistant viruses later in infection as observed in our present study. With the exception of the highly 2G12 sensitive viruses in patient H4, prevalence of 2G12 resistant viruses was already relatively high early in infection, and maintained during later stages of infection.

Antibodies b12, 2F5, and 4E10 are thought to target some of the most conserved regions of the viral envelope. It is therefore intriguing that BNab resistant virus variants emerge during the course of natural HIV-1 infection and this raises the question which mechanisms are responsible for their selection. The autologous NAb response has been shown to continuously select for escape variants which are resistant to circulating antibodies (Bunnik et al., 2008; Richman et al., 2003; Sagar et al., 2006; Wei et al., 2003). In patients H2, H3, and H5, the increasing resistance to HIVIg neutralization early in infection may reflect the escape from autologous NAbs with similar epitope specificities as the NAbs in HIVIg. However, a large proportion of the autologous neutralizing activity seems to be directed against the variable regions of the viral envelope, making it unlikely that escape from these antibodies would influence the sensitivity for certain BNAbs, such as the MPER-directed 2F5 and 4E10. Mutations in the V2 region have been associated with resistance against b12 (Mo et al., 1997; Pantophlet et al., 2002), indicating that escape from antibodies targeting the V2 loop may affect b12 sensitivity. However, the observation that b12 resistant virus variants emerged late in infection when the autologous NAb response has subsided (Bunnik et al., 2008), argues against the selection of these variants by NAb pressure. Alternatively, viral evolution in late stage disease, driven by reducing numbers of target cells and/or the absence of strong immune responses (Koning et al., 2003; Repits et al., 2005) may result in changes in the viral envelope that also affect the binding to neutralizing antibodies. In particular, adaptation to a more efficient CD4 usage at later disease stages may lead to an increased resistance against b12 as was observed in this study, as b12 competes with CD4 for attachment to the CD4 binding pocket. On the other hand, b12 sensitive virus variants (in patient H4), as well as 2F5 sensitive viruses (in patients H3, H4, and H5) persisted and coexisted with resistant variants. The continuous presence of BNAb sensitive virus variants in these patients suggests that the acquisition of BNAb resistance did not lead to a selective advantage and might have occurred randomly, rather than as a result of strong selective pressure.

As b12 recognizes a discontinuous conformational epitope (Pantophlet et al., 2002), it is difficult to analyze which mutations in the viral envelope contributed to resistance against this BNAb. Mutations in various regions of the envelope have been described to influence sensitivity to b12 neutralization (Duenas-Decamp et al., 2008; Mo et al., 1997; Pantophlet et al., 2002), although many of these changes seem to be isolate-specific. Indeed, in viruses from patient H5, we have identified a number of mutations in the envelope V1, V2 and V4 regions which appear to increase b12 resistance (Bunnik, manuscript in preparation), while these mutations were not found in late viruses from patients H3 and H4, who also displayed a b12 resistant phenotype.

In agreement with previous reports (Binley et al., 2004; Manrique et al., 2007), a good correlation was observed between the absence of one or more of the five potential N-linked glycosylation sites that may harbour the glycans of which the 2G12 epitope is composed and the viral inhibition by 2G12. Of the 117 virus variants that lacked one or more glycans, only 4 viruses were sensitive to 2G12 neutralization. The 14 viruses that contained an intact 2G12 epitope were all highly or moderately sensitive to 2G12 neutralization.

For 2F5, little sequence variation was observed in the core epitope, in line with a general sensitive neutralization phenotype of the viruses. Patient H1 was the only individual in which a substitution in the epitope (ELDQWA) of the virus evidently influenced its susceptibility to 2F5 neutralization. This K to Q substitution at position 665 has previously been described as a 2F5 escape mutation in vitro (Nakowitsch et al., 2005). It has been shown that resistance against 2F5 in vivo might be difficult to achieve and often results in variants with a reduced fitness (Manrique et al., 2007). Among 309 subtype B sequences in the Los Alamos database, 3.6% harboured a K665Q substitution (data not shown), which was the most frequently observed substitution among sequences which did not contain the wild-type K residue at position 665 (13.0% of all sequences). These data indicate that the K665Q substitution might be a relatively common pathway to escape from 2F5-like antibodies, although we cannot exclude that the mutation was introduced by random sequence variation.

In this study, we have used a maximum of five clonal HIV-1 variants per time point to assess the variation in neutralization sensitivity at that time point. Although it will be difficult to establish how representative each virus clone is in relation to the viral quasispecies, the use of clonal virus variants has clear advantages over the use of for example virus from bulk culture. The use of bulk virus will most likely underestimate the amount of variation within the quasispecies, as the neutralization sensitivity of a bulk virus population will largely be determined by the most neutralization resistant variant within that bulk. Moreover, the range in neutralization sensitivities of clonal HIV-1 variants in our study was very small at some time points, suggesting that when we do observe variation in sensitivity to Ab neutralization between clonal HIV-1 variants, this probably is a true reflection of variation in neutralization sensitivity within the viral quasispecies.

It has been suggested that in a PBMC-based assay, the presence of LPS in serum or antibody preparations may result in the suppression of HIV replication by chemokines that are released from monocyte-derived macrophages (MDM) in response to LPS (Verani et al., 1997), which can subsequently incorrectly be interpreted as antibody mediated neutralization. For our experiments, we used a single batch of pooled PBMC from 12 healthy blood donors. The observation that many viruses tested negative for neutralization by one or more of the four BNAbs suggests that our assay is not confounded by a specific viral inhibition as a result of the presence of LPS.

In conclusion, we have shown that, with a few exceptions, the earliest virus populations isolated from five typical progressors were most sensitive for neutralization by BNAbs, and that virus resistant to one or more BNAbs developed over the course of infection in most individuals. These BNAb resistant virus variants have most likely not been selected by antibody pressure, indicating that other selective processes may be involved. For vaccine design, it will be important to understand which mechanisms drive the selection of BNAb resistant virus variants.

Materials and methods

Patients and viruses

The patients in our present study were homosexual male participants of the Amsterdam Cohort Studies on HIV/AIDS (ACS) who seroconverted during active follow-up and who progressed to AIDS in the presence of CCR5-using (R5) HIV-1 variants only, as shown by absent virus replication in 3-monthly performed cocultures of patient PBMC and the MT2 cell line. For all virus variants studied here, CCR5 usage was predicted by the V3 loop sequence, and confirmed by the inability of these viruses to replicate in the MT2 cell line. For better

readability, patient identifiers were recoded as H1 (ACH19999), H2 (ACH19542), H3 (ACH18969), H4 (ACH19768) and H5 (ACH19659), which correspond to the identifiers used in a previous study (Bunnik et al., 2008). Clonal HIV-1 variants were obtained as previously described (Schuitemaker et al., 1992; Van 't Wout et al., 2008). For further study, we selected a maximum of five virus variants per patient per time point, among which were both rapidly and slowly replicating viruses. The viral replicative capacity was defined as the first day of detectable p24 production in the micro-culture after the start of the clonal virus isolation procedure. To prevent a change in neutralization sensitivity of the virus variants during in vitro culture, the number of peripheral blood mononuclear cell (PBMC) passages of viruses was kept to a minimum (Beaumont et al., 2004).

Neutralization assays

Primary HIV-1 were tested for their neutralization sensitivity against HIVIg (AIDS reagent program #3957) using a TZM-bl-based assay, and against broadly neutralizing monoclonal antibodies b12 (kindly provided by D. Burton), 2G12, 2F5, and 4E10 (Polymun Scientific, Vienna, Austria) using a PBMC-based assay. Both assays were performed in triplicate, using the same clonal HIV-1 variants.

(i) TZM-bl-based assay

To inhibit replication of the virus variants in TZM-bl cells, a final concentration of 1 μM indinavir (AIDS reagent program #8145) was added to Iscove's modified Dulbecco medium (Whitaker) supplemented with 10% fetal bovine serum (Hyclone), penicillin (Gibco Brl) (100 U/ml), streptomycin (Gibco Brl) (100 μg/ml), and DEAE dextran (37.5 μg/ml). From each virus isolate, a final inoculum of 20 $TCID_{50}$, as determined on TZM-bl cells, in a volume of 100 μl was incubated for 1 h at 37 °C with twofold serial dilutions of HIVIg (range 15.6–500 μg/ml). Subsequently, the mixtures of virus with antibody were added to 10^4 TZM-bl cells in 100 μl medium. After 48 h, the TZM-bl cells were washed once in 150 μl phosphate-buffered saline. Next, 25 μl freshly prepared luciferase substrate (0.83 mM ATP, 0.83 mM d-luciferin [Duchefa Biochemie B.V., Haarlem, The Netherlands], 18.7 mM $MgCl_2$, 0.78 μM $Na_2H_2P_2O_7$, 38.9 mM Tris [pH 7.8], 0.39% [vol/vol] glycerol, 0.03% [vol/vol] Triton X-100, and 2.6 μM dithiothreitol) was added and luminescence was measured for 1 s per well. For calculations, the background luciferase expression was subtracted from the relative light units (RLU) of the test wells. The percent neutralization was calculated by determining the reduction in luciferase expression in the presence of neutralizing agent compared to the cultures with virus only.

(ii) PBMC-based assay

Prior to the experiment, cryopreserved pooled PBMCs isolated from buffy coats obtained from 12 healthy seronegative blood donors by Ficoll-Isopaque density gradient centrifugation were thawn. The cells (5×10^6/ml) were stimulated for 3 days in IMDM supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), ciproxin (5 μg/ml), and phytohemagglutinin (PHA; Welcome) (5 μg/ml). From each virus isolate, a final inoculum of 20 $TCID_{50}$, as determined on the same pool of PBMC, in a volume of 100 μl was incubated for 1 h at 37 °C with threefold serial dilutions of monoclonal antibody (range 0.034–25 μg/ml). Subsequently, the mixtures of virus with antibody were added to 10^5 PHA-stimulated PBMCs in 50 μl medium, in the absence of PHA and supplemented with recombinant interleukin-2 (20 U/ml, Chiron Benelux, Amsterdam, The Netherlands) and polybrene (5 μg/ml; hexadimethrine bromide, Sigma, Zwijndrecht, The Netherlands). Virus production in culture supernatants on day 7 was analyzed by an in-house p24 antigen capture enzyme-linked immunosorbent assay (ELISA) (Tersmette et al., 1989). The percent neutralization was calculated by determining the reduction in p24 production in the presence of neutralizing agent compared to the cultures with virus only.

Sequence analysis

Env was amplified from DNA that was isolated from healthy donor PBMC that were infected in vitro with clonal HIV-1 variants. Env PCR products were subsequently sequenced as described previously (Beaumont et al., 2001; Boom et al., 1991; Quakkelaar et al., 2007). Nucleotide sequences of all virus clones per individual were aligned using ClustalW in the software package of BioEdit (Hall, 1999), and edited manually. The reference sequence HXB2 was included in the alignment to number each aligned residue according to the corresponding position in this reference sequence. Potential N-linked glycosylation sites were identified using N-Glycosite (Zhang et al., 2004) at the HIV database website (http://www.hiv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html).

Statistical analysis

For calculations and statistical analyses, viruses with IC_{50}s of >25 μg/ml (for monoclonal antibodies), or >500 μg/ml (for HIVIg) were assigned a value of 25 or 500, respectively. Statistical analyses were performed in SPSS 16 software package. Longitudinal changes in neutralization sensitivity were assessed using a Kruskal–Wallis test. Differences in susceptibility between virus variants isolated from two different time points were evaluated using the Mann–Whitney *U* test.

Nucleotide sequence accession numbers

All sequences of virus variants included in this study are available from GenBank (accession numbers EU743973 to EU744175).

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