

High prevalence of neutralizing activity against multiple unrelated human immunodeficiency virus type 1 (HIV-1) subtype B variants in sera from HIV-1 subtype B-infected individuals: evidence for subtype-specific rather than strain-specific neutralizing activity

Marit J. van Gils,¹ Diana Edo-Matas,¹ Becky Schweighardt,² Terri Wrin² and Hanneke Schuitemaker¹

Correspondence

Hanneke Schuitemaker
h.schuitemaker@amc.uva.nl

¹Department of Experimental Immunology, Landsteiner Laboratory Sanquin Research, and Center for Infection and Immunity (CINIMA), Academic Medical Center at the University of Amsterdam, Amsterdam, The Netherlands

²Monogram Biosciences, South San Francisco, CA, USA

It is assumed that an effective human immunodeficiency virus type 1 (HIV-1) vaccine should be capable of eliciting neutralizing antibodies. However, even the best antibodies known to date lack neutralizing ability against a significant proportion of primary HIV-1 variants and, despite great efforts, still no immunogen is available that can elicit humoral immunity which is protective against infection or disease progression. We tested sera from 35 participants in the Amsterdam Cohort Studies on HIV-1 infection, who were all infected with HIV-1 subtype B and therapy-naïve at the time of sampling, for neutralizing activity against a panel of 23 tier 2–3 HIV-1 variants, with a minimum of five HIV-1 variants per subtype (A, B, C and D). Strong cross-clade neutralizing activity was detected in sera from seven individuals. Strikingly, sera from 22 of 35 individuals (63%) neutralized three or more of the six tier 2–3 HIV-1 subtype B viruses in the panel. There was a strong correlation between neutralization titre and breadth in serum. Indeed, the IC₅₀ of sera with strong cross-clade neutralizing activity was significantly higher than the IC₅₀ of sera with cross-subtype B activity, which, in turn, had a higher IC₅₀ than sera with the lowest neutralization breadth. These results imply that humoral immunity, at least in HIV-1 subtype B-infected individuals, is often subtype-specific rather than strain-specific and that the breadth of neutralization is correlated with the titre of neutralizing activity in serum. Considering the difficulties in designing a vaccine that is capable of eliciting cross-clade neutralizing activity, subtype-specific vaccines may be explored as an interesting alternative.

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INTRODUCTION

Neutralizing antibodies (NAbs) are believed to be crucial for immunity against virus infections and are therefore considered an essential component of a human immunodeficiency virus type 1 (HIV-1) vaccine-elicited immune response (Walker & Burton, 2008). The development of an immunogen that is capable of eliciting NAbs is, however, challenged by the inaccessibility of conserved epitopes and the enormous sequence diversity of the viral envelope (McCutchan, 2000), which is the main target for NAbs. Indeed, the error-prone reverse transcriptase, the lack of proofreading and the extremely rapid virus-turnover rate are responsible for huge sequence variation, which can be as high as 10% already within the virus quasispecies in a

single individual (Gaschen *et al.*, 2002; Malim & Emerman, 2001; Shankarappa *et al.*, 1999). This high diversity has led to a classification of HIV-1 variants into distinct clades or subtypes, which are defined as groups of viruses that resemble each other more closely than viruses from other subtypes. The main (M) group is subdivided into subtypes A–K and different recombinant forms, which have different geographical distributions: subtype B, for instance, predominates in Europe, the Americas and Australia, whereas subtype C predominates in sub-Saharan Africa (Stebbing & Moyle, 2003). The viral envelope currently differs by up to 35% between subtypes and up to 20% within subtypes (Gaschen *et al.*, 2002; Hemelaar *et al.*, 2006; Taylor *et al.*, 2008). The enormity of this challenge can be put into perspective by comparison with the influenza vaccine,

where a diversity of <2% in amino acid changes can already cause failure in the cross-reactivity of the polyclonal response elicited by the vaccine (Gaschen *et al.*, 2002). It may therefore be put into question whether a single vaccine capable of eliciting NAb against all HIV-1 variants is feasible.

In addition to the high sequence diversity, the humoral immune response is thwarted by the inaccessibility of the relevant (conserved) epitopes. The inaccessibility of relevant epitopes on the HIV-1 envelope is due to a high level of glycosylation, occlusion within the oligomeric structure of the viral envelope and the fact that their formation occurs only after engagement of the viral envelope with CD4, when spatial constraints do not allow binding of relatively large immunoglobulins (Labrijn *et al.*, 2003). Despite viral mechanisms for evading humoral immunity, HIV-1 does elicit NAb in the natural course of infection. These, however, are considered to be mainly strain-specific, so are only capable of neutralizing autologous virus variants (Moog *et al.*, 1997) and their epitopes are therefore considered irrelevant for vaccine design.

Broadly neutralizing antibodies (BrNAbs) may bypass viral defence mechanisms, as they have the ability to neutralize HIV-1 variants from different subtypes (Binley *et al.*, 2004). Four well-known BrNAbs, b12, 2G12, 2F5 and 4E10, have been isolated from HIV-1-infected individuals. One of the current vaccine strategies is to design an immunogen that mimics the epitopes of these BrNAbs (Burton *et al.*, 2004). However, an effective vaccine would require additional epitope specificities, as a significant proportion (approx. 15%) of primary subtype A, B, C, D and CRF01-AE is resistant to neutralization by all four BrNAbs mentioned above (Binley *et al.*, 2004; Gray *et al.*, 2006; McKnight & Aasa-Chapman, 2007; Quakkelaar *et al.*, 2007; Richman *et al.*, 2003). The high sequence diversity between HIV-1 variants may underlie the incomplete coverage by BrNAbs. In that light, vaccine-elicited subtype-specific NAb may be the best alternative to BrNAbs. However, the existence of HIV-1 neutralization serotypes has been questioned (McKnight & Aasa-Chapman, 2007; Moore *et al.*, 2001).

Here, we studied the breadth of serum neutralizing activity in 35 HIV-1 subtype B-infected individuals. We found that sera from seven individuals had highly cross-clade neutralizing activity and that the majority of sera neutralized multiple unrelated subtype B HIV-1 variants, providing evidence for an HIV-1 subtype B neutralization serotype.

RESULTS

Prevalence of strong cross-clade HIV-1-specific neutralizing activity in patient sera

We studied sera from 35 participants in the Amsterdam Cohort Studies for the breadth and titre of HIV-1-specific

neutralizing activity. Serum samples were obtained between 24 and 33 months after the estimated day of seroconversion and all participants were therapy-naïve at this point. HIV-1-specific NAb activity was measured in a cell-based infectivity assay using recombinant viruses that carried a luciferase reporter gene and that were pseudotyped with envelope proteins from tier 2–3 HIV-1 subtype A, B, C and D. For comparison, five HIV-1 subtype B reference strains were additionally tested. To monitor neutralizing activity not mediated by antibodies directed against HIV-1 Env-specific antibodies, each plasma sample was also tested against a recombinant virus stock that was pseudotyped with amphotropic murine leukemia virus (aMLV) envelope proteins (gp70SU and p15TM). Typically, neutralization titres, expressed as the reciprocal dilution of plasma that established 50% inhibition (IC₅₀) of virus infection, were <40 for aMLV controls. No differences in neutralizing activity were observed between sera from long-term non-progressors (LTNPs) and progressors (cross-clade neutralizing activity in three of 20 LTNPs and four of 15 progressors; van Gils *et al.*, 2010). In all sera, neutralizing activity against the reference strains was observed (Tables 1–3).

Strong HIV-specific cross-clade neutralizing activity, defined as an IC₅₀ ≥ 100 for at least 50% of the tier 2–3 viruses from at least three different subtypes (so excluding the reference strains), was observed in sera from seven of 35 individuals (20%) (Table 1). Interestingly, sera from three of these individuals neutralized >80% of all tier 2–3 viruses in the panel with an IC₅₀ ≥ 100 (Table 1; patients 19298, 19642 and 19708).

Prevalence of sera with cross-reactive neutralizing activity against multiple HIV-1 subtype B variants, but less neutralizing activity to viruses from other subtypes

The sera from seven individuals with strong cross-clade neutralizing activity also neutralized five or six of the six tier 2–3 subtype B HIV-1 variants in the panel. Sera from the other 28 of 35 HIV-1 subtype B-infected individuals studied here lacked strong cross-clade neutralizing activity against HIV-1 variants from multiple subtypes, according to the definition described above. Interestingly, while sequence diversity between the envelope genes of the tier 2–3 HIV-1 subtype B variants in the panel, so again excluding the reference strains, varied by up to 12%, and while phylogenetic analysis did not reveal clustering of the viruses from this panel with autologous viruses of the different patients studied here (data not shown), sera from 26 of these 28 patients (93%) who lacked strong cross-clade neutralizing activity showed neutralizing activity against at least one of the six unrelated tier 2–3 HIV-1 subtype B variants in the panel (Tables 2 and 3). Strikingly, sera from 15 of these 28 patients (54%) neutralized three or more of the six unrelated tier 2–3 HIV-1 subtype B variants in the panel (Table 2). Interestingly, four of these

Table 1. Breadth and titre of HIV-1-specific neutralizing activity in sera from patients with strong cross-clade neutralizing activity

Patient IDs in italics are LTNPs. Titres <40 are indicated by –. Titres less than three times higher than the negative control are in parentheses. NA, Not applicable; ND, not determined.

Subtype/virus	Virus type	Origin	Patients with strong cross-clade neutralizing activity						
			19956	19298	19554	19708	19642	18969	19829
Tier 2–3 panel									
A									
MB_pA1	Primary	Uganda	72	174	100	284	130	112	94
MB_pA2	Primary	Uganda	42	129	–	210	51	–	133
MB_pA3	Primary	Uganda	88	245	215	520	203	671	309
94UG103	AIDS Repository	Uganda	88	254	373	423	258	106	–
92RW020	AIDS Repository	Rwanda	201	200	300	569	252	1118	935
B									
APV-16	Primary	USA	124	307	262	462	188	98	115
APV-20	Primary	USA	427	341	236	273	215	121	59
APV-9	Primary	USA	123	122	148	193	170	67	65
92BR020	AIDS Repository	Brazil	320	363	576	(63)	377	551	431
MB_pB1	Primary	USA	146	182	343	136	173	–	65
MB_pB2	Primary	USA	314	594	990	249	391	285	172
C									
MB_pC1	Primary	Europe	113	207	163	(49)	110	–	73
93IN905	AIDS Repository	India	225	–	67	114	85	182	48
IAVI_C22	AIDS Repository	Africa	42	60	72	72	(41)	–	–
MBC6	Primary	Africa	79	308	136	170	104	181	106
MBC3	Expanded in PBMC	Zimbabwe	149	499	372	292	214	892	1257
94IN11246-3	AIDS Repository	India	112	715	241	147	268	1335	1707
93MW960	AIDS Repository	Malawi	186	315	187	357	207	515	573
D									
MB_pD1	Primary	Uganda	200	694	189	172	187	–	–
MB_pD2	Primary	Uganda	103	365	209	153	100	73	–
MB_pD3	Primary	Uganda	77	95	88	88	–	95	–
92UG001	AIDS Repository	Uganda	187	232	97	165	161	–	–
93UG070	AIDS Repository	Uganda	195	271	204	209	121	–	–
Reference									
B									
1196	Reference strain	USA	512	ND	ND	285	491	919	956
BaL	Reference strain	USA	1247	1754	ND	2792	898	514	751
JRCFSF	Reference strain	USA	126	348	1096	708	261	142	67
NL4-3	Reference strain	USA	2409	1202	5239	1256	2771	1153	1166
SF162	Reference strain	USA	13936	ND	ND	ND	5343	4784	4075
Negative control									
aMLV	Murine leukemia	NA	–	–	–	–	–	–	–

patients (19250, 19559, 19663 and 19768) and also two patients with strong cross-clade neutralizing activity (18969 and 19829) showed the same breadth of neutralization against subtype B and subtype C viruses, with even higher neutralizing titres against the subtype C variants than against the subtype B variants.

The breadth of neutralizing activity against viruses from the other three subtypes was significantly lower, in agreement with the fact that these sera did not have strong cross-clade neutralizing activity. These data show that, apart from the seven sera with strong cross-clade neutralizing activity, the majority of sera had neutralizing

activity against multiple and diverse subtype B HIV-1 variants. Indeed, of the total of 35 individuals, 22 (63%) had neutralizing activity against at least three of the tier 2–3 subtype B viruses in the panel.

Correlation between titre and breadth of HIV-1-specific neutralizing activity in serum

Characteristics of heterologous HIV-1-specific neutralizing serum reactivity are not known in great detail. Here, we observed a strong correlation between the titre of neutralizing activity and the number of different viruses that were neutralized by a serum (Fig. 1). Indeed, for

Table 2. Breadth and titre of HIV-1-specific neutralizing activity in sera from patients with cross-reactive but mainly subtype B-specific neutralizing activity

Patient IDs in italics are LTNPs. Titres <40 are indicated by –. Titres less than three times higher than the negative control are in parentheses. For virus type and origin, see Table 1.

Subtype/virus	Patients with cross-reactive but mainly subtype B-specific neutralizing activity														
	19250	19559	19663	19768	19542	18971	19999	19383	18829	19335	19789	19843	19417	19334	19342
Tier 2–3 panel															
A															
MB_pA1	46	61	–	78	47	49	63	–	–	–	–	–	–	–	–
MB_pA2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
MB_pA3	166	142	126	111	–	124	335	–	–	–	–	40	–	–	–
94UG103	–	–	–	–	–	40	–	–	–	–	–	–	–	–	–
92RW020	272	143	441	187	58	86	83	–	–	–	–	54	44	–	–
B															
APV-16	82	69	116	59	44	47	68	58	–	46	61	49	46	40	51
APV-20	89	94	105	–	96	56	84	53	67	106	54	50	54	88	53
APV-9	41	–	53	–	258	42	–	–	67	–	–	–	–	–	–
92BR020	290	220	412	77	62	96	52	77	80	95	79	93	64	81	62
MB_pB1	49	93	71	78	–	–	–	41	–	–	41	–	–	–	–
MB_pB2	97	108	106	200	81	82	–	80	85	64	–	–	56	–	–
C															
MB_pC1	40	45	–	44	71	–	–	–	–	–	–	–	–	–	–
93IN905	149	195	221	–	56	–	–	–	–	–	–	–	–	–	–
IAVI_C22	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
MBC6	197	74	59	115	–	–	–	–	–	–	–	–	–	–	–
MBC3	511	290	677	255	141	(55)	–	–	80	–	–	–	–	65	51
94IN11246-3	380	340	607	64	–	–	–	–	–	–	–	–	–	–	–
93MW960	207	273	267	107	–	77	76	47	–	48	49	–	–	–	–
D															
MB_pD1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
MB_pD2	146	87	–	–	54	–	–	–	–	–	–	–	–	–	–
MB_pD3	–	–	–	–	–	–	41	–	–	–	–	–	–	–	–
92UG001	153	53	45	–	–	–	–	–	–	–	–	–	–	–	–
93UG070	189	65	–	43	–	–	–	–	–	–	–	–	–	–	–
Reference															
B															
1196	546	270	474	135	256	218	284	102	148	175	179	70	164	91	133
BaL	820	328	815	358	297	284	104	219	385	243	157	123	478	100	260
JRCFSF	–	85	128	–	–	57	72	–	–	–	–	83	74	–	–
NL4-3	2654	936	849	338	727	563	371	1645	1909	936	668	480	948	676	1312
SF162	11257	2307	3624	1736	4016	2781	1157	3770	4019	3732	2317	1497	3209	1711	3066
Negative control															
aMLV	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

neutralization of each individual virus in the panel of tier 2–3 HIV-1 subtype B viruses, the mean IC₅₀ values were significantly higher for sera that had strong cross-reactive neutralizing activity against viruses from different subtypes (Fig. 1a, empty bars) compared with sera with cross-reactive neutralizing activity against multiple subtype B variants, but not against viruses from other subtypes (Fig. 1a, grey-shaded bars). Additionally, sera from the latter group had, in turn, a significantly higher mean neutralizing titre against four of the six tier 2–3 subtype B HIV-1 variants in the panel (92BR020, APV-16, APV-20 and MB_pB1) compared with the mean neutralizing titres in the 13 patient sera that

neutralized up to two of the HIV-1 subtype B viruses in the panel (Fig. 1a, diagonally hatched bars).

The mean neutralizing titres in the patient sera studied here were higher for some of the reference viruses that were used in this study (1196, BaL, JRCFSF, NL4-3 and SF162; Fig. 1b), in agreement with the generally higher neutralization sensitivity of these viruses. Interestingly, also for these reference strains, we observed the same pattern between neutralization breadth and titre. Indeed, the mean neutralizing titre of the seven sera with strong cross-clade neutralizing activity (Fig. 1b, empty bars) was significantly

Table 3. Breadth and titre of HIV-1-specific neutralizing activity in sera from patients with absent cross-reactive neutralizing activity. Patient IDs in italics are LTNPs. Titres <40 are indicated by -. Titres less than three times higher than the negative control are in parentheses. ND, Not determined. For virus type and origin, see Table 1.

Subtype/virus	Patients with absent cross-reactive neutralizing activity												
	19992	19943	19933	18789	19974	19984	19874	19659	19291	19951	18880	19552	19406
Tier 2-3 panel													
A													
MB_pA1	-	-	-	-	-	-	-	-	-	-	-	-	-
MB_pA2	-	-	-	-	-	-	-	-	-	-	-	-	-
MB_pA3	-	-	-	-	-	-	-	-	-	-	-	-	-
94UG103	-	(42)	-	-	-	-	-	-	-	-	-	-	-
92RW020	74	-	119	42	-	-	-	-	-	-	-	-	-
B													
APV-16	(46)	(40)	42	-	(47)	-	-	-	41	57	-	-	-
APV-20	60	(55)	-	83	60	(54)	49	43	-	-	41	-	-
APV-9	-	-	-	71	-	-	-	-	-	-	-	-	-
92BR020	123	46	-	-	91	76	96	54	41	-	-	-	-
MB_pB1	-	-	-	-	-	-	-	-	-	-	-	-	-
MB_pB2	-	132	-	-	-	-	-	-	-	-	-	-	-
C													
MB_pC1	-	-	-	-	-	-	-	-	-	-	-	-	-
93IN905	68	-	-	-	-	-	-	-	-	-	-	-	-
IAVI_C22	-	-	-	-	-	-	-	-	-	-	-	-	-
MBC6	-	-	92	-	-	-	-	-	-	-	-	-	-
MBC3	60	-	-	-	-	55	-	-	-	-	-	-	-
94IN11246-3	-	-	-	-	-	-	-	-	-	-	-	-	-
93MW960	-	(49)	-	-	(48)	-	-	-	-	-	-	-	-
D													
MB_pD1	-	-	-	-	-	-	-	-	-	-	-	-	-
MB_pD2	-	-	-	-	-	-	-	-	-	-	-	-	-
MB_pD3	-	-	-	-	-	-	-	-	-	-	-	-	-
92UG001	-	106	-	-	-	-	-	-	-	-	-	-	-
93UG070	-	74	-	-	-	-	-	-	-	-	-	-	-
Reference													
B													
1196	161	73	-	76	150	218	124	98	47	100	44	48	58
BaL	309	93	-	47	237	216	256	218	71	156	45	54	-
JRCSF	(54)	-	-	-	(47)	228	-	-	-	-	-	-	-
NL4-3	777	522	44	141	916	958	360	608	177	598	409	187	121
SF162	2713	2212	264	647	3414	1530	4353	1900	1067	2385	ND	497	949
Negative control													
aMLV	-	-	-	-	-	-	-	-	-	-	-	-	-

higher for each individual reference virus than the mean neutralizing titre in the 15 sera with subtype B-specific cross-reactive neutralizing activity (Fig. 1b, grey-shaded bars), while the mean neutralizing titres in these sera were again higher than the mean neutralizing titre in the 13 sera that lacked cross-reactivity (Fig. 1b, diagonally hatched bars). For JRCSF, a tier 2 reference strain with a known neutralization-resistant phenotype (Moore *et al.*, 1995), the mean neutralizing titre in the 15 sera with cross-subtype B activity was similar to the mean titre in the 13 sera that lacked cross-reactive neutralizing activity (Fig. 1b).

The neutralizing titres against viruses of subtypes A, C and D also showed a correlation with the neutralization breadth

against these viruses, albeit that the differences in titres between groups of sera with strong cross-clade neutralizing activity, cross-subtype B neutralizing activity or almost absent neutralizing activity were less strong (data not shown).

DISCUSSION

All vaccines that provide protection against virus infections elicit at least a potent humoral immune response (Pantaleo & Koup, 2004). In line, HIV-1 vaccine research is aiming for an immunogen in which epitopes for BrNABs are present (Burton *et al.*, 2004). This is a challenging task, as

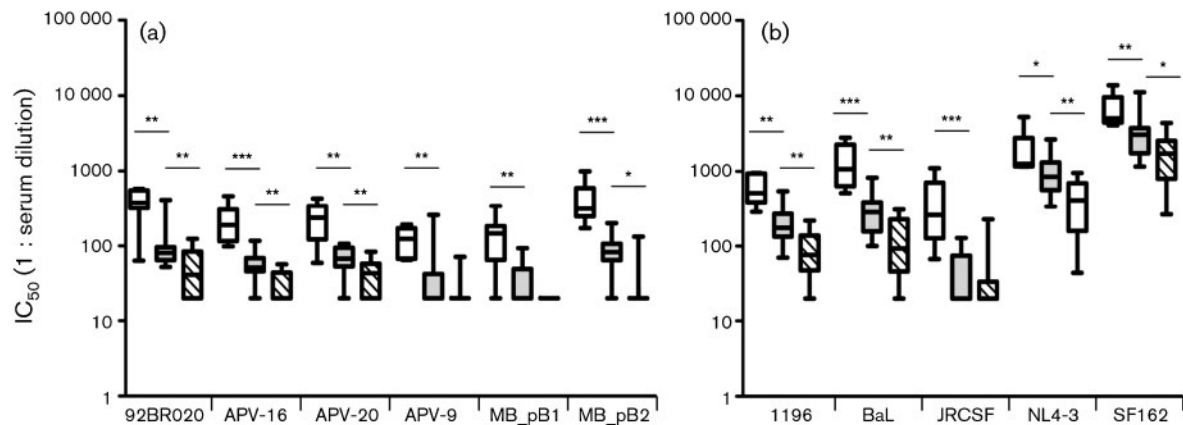


Fig. 1. Correlation between titre and breadth of HIV-1-specific neutralizing humoral immunity in sera of HIV-1-infected individuals. Mean neutralizing titre of sera in defined groups, according to their ability to neutralize the tier 2–3 viruses from the panel, against (a) six unrelated tier 2–3 subtype B HIV-1 variants and (b) five subtype B reference strains. Patient sera were grouped based on neutralizing activity against the tier 2–3 viruses, excluding neutralizing activity against the reference strains: strong cross-clade neutralizing activity (Table 1; $\geq 50\%$ of viruses per subtype with $IC_{50} \geq 100$ for at least three subtypes, $n=7$), cross-reactive neutralizing activity against multiple subtype B variants but minimal neutralizing activity against other subtypes (Table 2; $\geq 50\%$ of subtype B viruses neutralized, $n=15$) or absent cross-reactive neutralizing activity, (Table 3; $n=13$). Serum neutralizing titres required for 50% inhibition of the tier 2–3 HIV-1 subtype B virus variants in the panel were calculated. Empty bars represent sera with strong cross-clade neutralizing activity ($n=7$); grey-shaded bars represent sera with cross-reactive neutralizing activity against multiple subtype B variants but minimal reactivity against viruses from other subtypes ($n=15$); diagonally hatched bars represent sera that lack cross-reactive neutralizing activity ($n=13$). Neutralizing titres are expressed as the reciprocal of the plasma dilution that inhibited virus infection by 50%. Significant differences between the three groups are indicated: * $P<0.05$; ** $P<0.01$; *** $P<0.001$ (Mann–Whitney U test).

the HIV-1 envelope has evolved towards a structure in which the relevant epitopes are absent in the native protein, occluded in the oligomeric structure and/or covered by *N*-linked glycosylation sites. In addition, the HIV-1 envelope gene is highly variable. This variation, which can be up to 35% between different subtypes (Gaschen *et al.*, 2002; Hemelaar *et al.*, 2006; Taylor *et al.*, 2008), makes it unlikely that a single vaccine will be capable of eliciting a humoral immune response that would cover protection against all possible variants. Indeed, even the best BrNAbs known to date do not neutralize all of the circulating HIV-1 variants (Binley *et al.*, 2004; Gray *et al.*, 2006; McKnight & Aasa-Chapman, 2007; Quakkelaar *et al.*, 2007; Richman *et al.*, 2003). Most HIV-1-infected individuals mount an HIV-1-specific humoral immune response, but these antibodies are considered strain-specific, as neutralizing activity is assumed to be limited to the autologous virus strain. Indeed, the majority of HIV-1-infected individuals do not develop cross-clade neutralizing activity that is capable of neutralizing HIV-1 variants from different subtypes (Li *et al.*, 2007; Piantadosi *et al.*, 2009; Sather *et al.*, 2009). However, cross-reactive neutralization of different HIV-1 variants of the same subtype has received only little attention.

The findings of our present study suggest that subtype-specific differences in HIV-1 neutralization may exist, similar to what is known for influenza virus (Karlsson

Hedestam *et al.*, 2008; Webster *et al.*, 1992). Overall, we observed that sera from HIV-1 subtype B-infected individuals had stronger neutralizing activity against multiple unrelated HIV-1 subtype B variants with substantial sequence diversity in their envelopes than against HIV-1 variants from subtypes A, C and D. However, sera from four patients with neutralizing activity against multiple subtype B variants and from two patients with strong cross-clade neutralizing activity had higher neutralizing titres against the subtype C variants in our panel than against the variants from the other subtypes, including subtype B. This may suggest that at least some of the epitopes on the envelope of subtype B variants that elicited cross-clade neutralizing activity may be even better exposed on subtype C variants.

Obviously, it remains to be established whether this observation also holds true for sera from individuals infected with other HIV-1 subtypes. Other studies have not provided evidence for HIV-1 subtype-specific differences in HIV-1-neutralizing activity in serum (Kostrikis *et al.*, 1996; Moore *et al.*, 2001). However, these studies were performed with only a limited number of HIV-1 variants and sometimes with a pool of patient sera in which different neutralizing epitope specificities may have been mixed. Moreover, these studies focused strongly on BrNAbs that, by definition, neutralize HIV-1 variants from different subtypes. Although not emphasized specifically by

the authors, some previous reports do include data showing that neutralizing activity in patient sera was stronger against viruses that were from the same subtype as the autologous virus (Binley *et al.*, 2004; Brown *et al.*, 2008; Simek *et al.*, 2009).

The exact nature of the epitopes at which cross-clade neutralizing activity and subtype-specific cross-reactive neutralizing activity is directed remains to be established. It was reported recently that cross-clade neutralizing activity is not directed only against the conserved regions of the envelope, such as the CD4-binding site (Dhillon *et al.*, 2007; Doria-Rose *et al.*, 2009; Guan *et al.*, 2009; Li *et al.*, 2007; Sather *et al.*, 2009; Scheid *et al.*, 2009) or the V3 loop (Li *et al.*, 2009). It is likely that epitopes that are less well-conserved between subtypes, but conserved within a subtype, are capable of eliciting subtype-specific cross-reactive neutralizing activity. Alternatively, the neutralizing activity is mediated by antibodies directed against the V3 loop, similar to the HIV-1 subtype B-specific neutralizing activity of the well-characterized monoclonal antibody 446-52D. This NAb recognizes a GPxR motif that is very well-conserved in the V3 loop of subtype B HIV-1 variants (Conley *et al.*, 1994).

The observation that subtype-specific neutralizing activity in serum may exist can provide a new lead in HIV-1 vaccine development. Indeed, the high sequence diversity between HIV-1 variants of different subtypes may stand in the way of the development of a single vaccine capable of eliciting neutralizing humoral immunity against all circulating HIV-1 variants. Obviously, this approach may be considered once a successful protein vaccine has been developed, which is a major challenge in itself.

Interestingly, we observed relatively strong cross-reactive neutralizing activity against multiple subtype B variants in sera from 63% of subtype B-infected individuals studied here, suggesting that the epitopes that have elicited these humoral responses are present and accessible on natural HIV-1 variants. Although HIV-1 may escape rapidly from this antibody pressure (Bunnik *et al.*, 2008), escape may be prevented if a vaccine elicits sterilizing immunity that is capable of preventing virus replication completely.

We have also observed that the ability of serum to neutralize different viruses is related directly to the neutralization titre in serum (modelled in Fig. 2). Although this finding does not exclude that highly potent antibody specificities may exist at an average concentration in serum, as was reported recently for two novel cross-clade NAbs, PG9 and PG16 (Walker *et al.*, 2009), it may imply that sera with highly cross-clade neutralizing ability in general harbour multiple epitope specificities or that a high quantity of a single antibody specificity is more potent, even against unrelated HIV-1 variants. This observation indicates that, in general, optimal boosting during vaccination to increase the antibody titre elicited by a

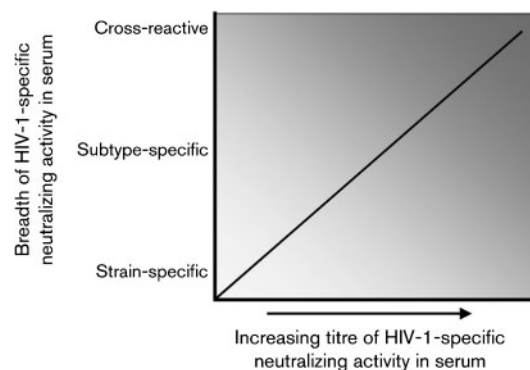


Fig. 2. Correlation between neutralizing breadth and titre in serum. On the *x*-axis, an increasing neutralization titre is suggested; on the *y*-axis, an increasing breadth of the response in three categories is depicted. The line represents the association between titre and breadth; increasing depth of shading in the background shows increasing potency of neutralizing activity.

future vaccine may also increase the breadth of the neutralizing activity significantly.

In conclusion, we have found evidence for subtype-specific neutralizing activity and a positive correlation between the titre and breadth of neutralizing activity in patient sera. The design of improved adjuvants that can optimize humoral immune responses, in combination with potentially subtype-specific epitopes, may thus provide new leads on the way to a potent HIV-1 vaccine. Developing and administering multiple HIV vaccines is far less ideal than having a single vaccine that would cover all circulating HIV variants. However, design and delivery of a single vaccine that is capable of eliciting potent and cross-clade neutralizing immunity against HIV-1 have not yet been successful. Although we realize that any vaccine approach will probably have to deal with the complexity of the HIV-1 envelope molecule and the difficulty to mimic it as an immunogen, based on our data we suggest that the approach of subtype-specific vaccines may be worthwhile to consider in current strategies.

METHODS

Patients. The study group consisted of LTNP (defined as HIV-1-infected individuals who have ≥ 10 years of asymptomatic follow-up with stable CD4⁺ cell counts that were still >400 cells μl^{-1} in the ninth year of follow-up) and progressors [HIV-1-infected individuals who progressed to AIDS within 7 years after (imputed) seroconversion] who were all participating in the Amsterdam Cohort Studies on HIV and AIDS in homosexual men. All individuals were infected with HIV-1 subtype B and were either seropositive at entry to the cohort studies (seroprevalent cases with an imputed seroconversion date on average 18 months before entry to the cohort; van Griensven *et al.*, 1989; Mascola *et al.*, 2005) or seroconverted during active follow-up in the cohort studies. None of the participants received combination anti-retroviral therapy during the sampling period; samples were obtained on average at 28 months (range, 24–33 months).

The Amsterdam Cohort Studies are conducted in accordance with the ethical principles set out in the Declaration of Helsinki and written consent was obtained prior to data collection from each participant. The study was approved by the Academic Medical Center institutional medical ethics committee.

Viruses. Sera from all 35 patients were tested for neutralizing activity in a pseudovirus assay developed by Monogram Biosciences. The tier 2–3 virus panel (Table 1) that we used for determining cross-neutralizing activity in serum consisted of HIV-1 pseudoviruses from subtypes A ($n=5$), B ($n=6$), C ($n=7$) and D ($n=5$) and included recently transmitted isolates and moderately neutralization-sensitive and -resistant primary HIV-1 variants, based on previously determined neutralization sensitivities to subtype B sera and mAbs b12, 2G12 and 4E10 (Binley *et al.*, 2004; Schweighardt *et al.*, 2007; Simek *et al.*, 2009). In addition, five subtype B HIV-1 reference strains were included (1196, BaL, JRCSF, NL4-3 and SF162; AIDS Repository, NIH, Bethesda, MD, USA). Pseudotyped virus particles were produced by cotransfecting HEK293 cells (AIDS Repository, NIH) with an expression vector carrying the patient-derived gp160 gene (eETV) and an HIV-1 genomic vector carrying a luciferase reporter gene (pRTV1.F-lucP.CNDO- Δ U3). Forty-eight hours after transfection, pseudovirus stocks were harvested and small aliquots were tested for infectivity by using U87 target cells (a gift from N. Landau, Department of Microbiology, New York University School of Medicine, NY, USA) expressing CD4, CCR5 and CXCR4. Pseudovirus stocks were then diluted to titres that, as measured by relative light units, fell within a range known to yield reproducible IC_{50} values.

Neutralization assay. A recombinant virus assay involving a single round of virus infection was used to measure neutralization (Petroopoulos *et al.*, 2000; Richman *et al.*, 2003). Diluted pseudoviruses were incubated for 1 h at 37 °C with serial dilutions of serum, after which the U87 target cells were added. The ability of patient sera to neutralize virus infection was assessed by measuring luciferase activity 72 h after virus inoculation in comparison to a control infection with a virus pseudotyped with the murine leukemia virus envelope (aMLV).

Neutralization titres are expressed as the reciprocal of the plasma dilution that inhibited virus infection by 50% (IC_{50}). Neutralization titres were considered positive if they were three times greater than the negative aMLV control.

Statistical analyses. Statistical analyses were performed by using the SPSS 16 software package. Neutralization titres, expressed as the reciprocal of IC_{50} , and the number of viruses that were neutralized were not distributed normally. Therefore, the non-parametric Kruskal–Wallis test and Mann–Whitney U test were used to compare the neutralization titres between sera that had strong cross-clade neutralizing activity, cross-subtype B-specific neutralizing activity only or no cross-reactive neutralizing activity at all. For the calculation of IC_{50} values for viruses that were not inhibited by the 1:40 serum dilution, we assumed that 50% inhibition would have occurred at a 1:20 serum dilution. A result was considered significant when the P -value was <0.05 .

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