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Virology 347 (2006) 36 – 51
VIROLOGY
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Neutralization and infectivity characteristics of envelope glycoproteins from human immunodeficiency virus type 1 infected donors whose sera exhibit broadly cross-reactive neutralizing activity

Fatim Cham [a,b,*], Peng Fei Zhang [a], Leo Heyndrickx [b], Peter Bouma [a], Ping Zhong [e,1], Herman Katinger [c], James Robinson [d], Guido van der Groen [b], Gerald V. Quinnan Jr. [a]

[a] *Uniformed Services University of the Health Sciences, Bethesda, MD 20814, USA*
[b] *Institute of Tropical Medicine, Antwerp, Belgium*
[c] *Institute of Applied Microbiology, University of Life Sciences, Vienna, Austria*
[d] *Department of Pediatrics, Tulane University, New Orleans, LA 70112, USA*
[e] *New York Veterans Administration Medical Center, New York, NY 01116, USA*

Received 22 June 2005; returned to author for revision 12 October 2005; accepted 8 November 2005
Available online 27 December 2005

Abstract

In this study, we tested the hypothesis that donors with broadly cross-reactive HIV-1 neutralizing (BCN) sera are infected with viruses encoding envelope glycoproteins (Envs) with unusual immunogenic properties. Cloned *env* genes were from samples of donors previously identified as having BCN antibodies (BCN donors) and from other donors not known to have such antibodies (non-BCN donors). Neutralization properties of viruses pseudotyped with BCN and non-BCN Envs were determined using BCN, non-BCN sera and broadly cross-neutralizing monoclonal antibodies (Mabs). BCN sera neutralized with higher frequency and geometric mean titers than non-BCN sera. Viruses pseudotyped with BCN Envs were mostly resistant to neutralization by anti-gp120 Mabs but tended to be more sensitive to the anti-gp41 Mabs, 2F5 and 4E10 than non-BCN Env-pseudotyped viruses. Sequence analysis of clones obtained from sequential samples of two BCN donors revealed respective 2F5 epitope mutations T662A and K665T. The K665T mutation evolved as the predominant genotype in the respective donor, consistent with an escape mutation event. The A662T mutation reduced sensitivity to 4E10, as well as 2F5 and homologous sera, consistent with neutralization escape mutation and targeting of the 2F5 epitope region by the serum. Our study suggests that viruses infecting these BCN donors encoded Envs that may have been unusually competent for induction of antibodies against the membrane proximal epitope region (MPER) of gp41, and these Envs may be useful vaccine components.

Keywords: HIV-1 envelope glycoprotein; Pseudovirions; Neutralization; Membrane proximal epitope region

Introduction

The definition of immunogenic epitopes on the HIV-1 envelope glycoprotein (Env) that can induce broadly cross-reactive neutralizing (BCN) antibodies remains an enigma. Efforts to develop vaccines that induce BCN antibodies have progressed slowly owing to HIV-1's ability to elude the host immune responses. The number and positioning of large clusters of N-linked glycan moieties and masking of conserved epitopes in the receptor and coreceptor-binding sites and in gp41 limit access of neutralizing antibodies to potentially important epitopes on the viral Env (Kwong et al., 2002; Labrijn et al., 2003; Scanlan et al., 2002, 2003; Wei et al., 2003; Zwick et al., 2001). Additionally, HIV-1 utilizes antigenic variation and mimicry of self antigens to thwart production of effective neutralizing antibodies (Haynes et al., 2005; Moore et al., 2001; Nabel, 2005).

Despite HIV's immune evasion tactics, reported findings of BCN antibodies in sera of infected patients indicate that HIV-1 strains share cross-reactive neutralization epitopes and humans

* Corresponding author. Mailing address: Department of Preventive Medicine and Biometrics, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814, USA. Fax: +1 301 295 3860.
E-mail address: Fcham@usuhs.mil (F. Cham).
[1] Current address: The Shanghai Municipal Center for Disease Control and Prevention, Shanghai, China.

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doi:10.1016/j.virol.2005.11.019

have the capacity to respond to these epitopes (Beirnaert et al., 2000; Donners et al., 2002; Nyambi et al., 1996; Quinnan et al., 1999; Vujcic and Quinnan, 1995; Zhang et al., 1999). Moreover, a number of cross-reactive neutralization epitopes are targeted by monoclonal antibodies (Mabs) isolated from infected patients (Burton et al., 1994; Moulard et al., 2002; Muster et al., 1994; Purtscher et al., 1994; Scanlan et al., 2003; Zhang et al., 2004; Zwick et al., 2001). The Mab IgG1 b12 and the antibody fragments (Fab) X5, 17b and 48d neutralize some HIV-1 isolates by targeting epitopes in the CD4 and coreceptor binding sites, respectively (Burton et al., 1994; Kwong et al., 2002; Labrijn et al., 2003; Moulard et al., 2002; Thali et al., 1991; Wyatt et al., 1995). The only potent broadly cross-reactive Mab reported to target a heavily glycosylated region of gp120 is 2G12 (Sanders et al., 2002; Scanlan et al., 2002, 2003). The Mab 447-52d recognizes a 14-mer epitope, KRIHIGPGRAFYTT, that forms a conserved type II β turn motif at the tip of the V3 loop of X4 and R5 isolates (Kwong, 2004; Sharon et al., 2003). The anti-gp41 Mabs 2F5 and 4E10 target highly conserved epitopes located near the C-terminal end of the gp41 ectodomain (Cardoso et al., 2005; Haynes et al., 2005; Ofek et al., 2004; Trkola et al., 1995; Zwick et al., 2001, 2004). Most recently, the novel antibodies, m14 and m18, which target the CD4bs and epitopes between the CD4 and coreceptor binding sites, respectively, have been shown to neutralize some primary isolates (Zhang et al., 2003, 2004). Studies using combinations of some of these Mabs, including b12, 2G12, F105, 4E10 and 2F5, have resulted in protection against intravenous and/or mucosal simian–human immunodeficiency virus (SHIV) challenge in monkeys (Li et al., 1997, 1998; Ruprecht et al., 2003). In addition, 2F5 and/or 4E10 plus 2G12 immunotherapy have resulted in reduced viremia in established HIV-1 infection or delay of HIV-1 rebound in individuals undergoing interrupted antiretroviral treatment (ART) (Stiegler et al., 2002; Trkola et al., 2005). Epitopes targeted by certain of these Mabs may be candidates for induction of BCN antibody responses.

Despite knowledge of the target epitopes of most of these potent antibodies, construction of vaccines that can induce broad cross-reactive antibodies has been a difficult challenge (Burton et al., 2004b; McGaughey et al., 2004; Muster et al., 1994; Nabel, 2005; Pantophlet and Burton, 2003; Saphire et al., 2003; Selvarajah et al., 2005). Nonetheless, immunization of small animals and macaques with a CD4-independent Env immunogen designated as strain R2, isolated from a donor whose serum possesses BCN activity (de Souza et al., 1997; Quinnan et al., 1999; Vujcic and Quinnan, 1995; Zhang et al., 2002), resulted in induction of antibodies that neutralized some heterologous primary HIV-1 strains (Dong et al., 2003; Quinnan et al., 2005). The titer of neutralizing antibodies correlated with protection of macaques against intravenous challenge with heterologous $SHIV_{DH12R,\ Clone\ 7}$ (Quinnan et al., 2005). The experience with R2 suggests that Envs from donors with HIV-1 BCN antibodies may be useful for induction of BCN antibodies in vaccinated animals or people.

Sera collected from approximately 10% of donors enrolled in cohort studies conducted at the Institute of Tropical Medicine (ITM), Belgium were reported to possess BCN activity (Beirnaert et al., 2000; Donners et al., 2002). BCN activity was defined as neutralization of three key primary isolates, namely, MN (subtype B), VI 525 (mixture of two viruses of Env subtypes A and G) and CA 9 (Group O) (Beirnaert et al., 2000) or strains CA 4 (subtype F), CA 13 (subtype H) and VI 686 (group O) (Donners et al., 2002). The BCN sera derived from donors infected with diverse HIV-1 subtypes neutralized either set of key isolates as well as panels of HIV-1 strains of multiple subtypes. The donors included in the ITM studies were European and Africans and were infected with diverse subtypes of HIV-1. The donors found to have BCN antibodies were mostly African females infected with diverse subtypes (Table 1) (Beirnaert et al., 2000; Donners et al., 2002; Nyambi et al., 2000).

We hypothesized that those HIV-1 patients from the ITM cohorts with BCN antibodies may harbor virus strains that encode Env proteins that are exceptionally competent for induction of BCN antibodies and that the reasons for the broad immunogenicity of the Envs would be reflected in neutralization characteristics of the Envs. To test our hypothesis, we cloned *env* genes and characterized the neutralization properties of the encoded Envs from six donors defined as having BCN antibodies (BCN donors) in the ITM studies and from 11 donors not characterized as having BCN antibodies (non-BCN

Table 1
Characteristics of BCN and non-BCN donors and envelope pseudotypes

	Donors	Env subtype	Origin	Sampling year	Luciferase units[a]
	R2	B	North America	1989	539,957
BCN	VI 423	B	Europe	1990	60,102
	VI 843	B	Europe	1993	99,690
	14/00/4	F1	Africa	1994	3,056,071
	VI 1249	CRF01_AE	Asia	1994	238,477
	24/00/4	CRF02_AG	Africa	1994	1,343,945
	VI 1793	CRF06_cpx	Africa	1996	769,105
Non-BCN	92RW20.5	A	Africa	1992	154,846
	NYU1423	A	Africa	2000	1,124,994
	VI 1273	B	Europe	1996	343,540
	VI 1399	B	Europe	1994	1,483,216
	MACS4	B	North America	1994	1,594,686
	GXC-14	C	Asia	N/A	1,527,941
	Z2Z6	D	Africa	1985	693,122
	93BR029	F1	South America	1993	142,795
	GXE-14	CRF01_AE	Asia	N/A	652,103
	NYU1026	CRF02_AG	Africa	2002	683,004
	CA1	CRF11_cpx	Africa	1993	4,225,479

GenBank accession numbers are as follows: VI 1793: DQ313254; VI 843: DQ313250; VI 423: DQ313249; 14/00/4: DQ313239; 24/00/4: DQ313242;VI 1249: DQ313251; NYU1423: DQ313248; VI 1273: DQ313252; VI 1399: DQ313253; MACS4 (4/116): DQ313246; NYU1026: DQ313247; 93BR029: AF005495; CA1: AJ277823. Sequences which have been submitted previously are: R2: AF128126; GXC-14: AY217546; GXE-14: AY217545; and Z2Z6: K03458.

[a] Values indicate luminescence reading in HOS $CD4^+CCR5^+$ cells infected with undiluted viruses pseudotyped with the indicated Env, except for VI 1249 and Z2Z6, which were tested for infectivity in HOS $CD4^+CXCR4^+$.

donors). The Env subtypes of viruses infecting BCN and non-BCN patients belonged to HIV-1 subtypes A, B, C, D, F, CRF01_AE, CRF02_AG, CRF06_cpx and CRF11_cpx. The neutralization characteristics were determined using viruses pseudotyped with cloned Envs. Neutralization was tested using autologous sera, heterologous sera, soluble CD4 (sCD4) and a panel of Mabs directed against conformation-dependent epitopes in gp120 and gp41. Our results suggest that certain of these Env may have unusual properties associated with neutralizing antibody responses to important membrane proximal neutralization epitopes in the gp41 ectodomain.

Results

Infectivity and serum neutralization of viruses pseudotyped with functional HIV-1 env genes

For each donor, approximately 10% of the Env clones obtained mediated infection of Human Osteosarcoma (HOS) cells expressing CD4 and either CCR5 or CXCR4, as measured by luciferase activity. Among those that were functional, the majority had similar levels of infectivity (data not shown), and a clone with the highest apparent infectivity was selected for further characterization. Characteristics of Env clones obtained in this study are shown in Table 1. The Envs generated in this study were CCR5-tropic, except for Z2Z6 and VI 1249, which displayed dual-tropism for CCR5 and CXCR4 (Quinnan et al., 1999). The luciferase units detected in $CCR5^+$ and $CXCR4^+$ HOS cells infected with undiluted virus pseudotyped with Env Z2Z6 were 113, 379 and 693,122 respectively. The luciferase units detected in $CCR5^+$ and $CXCR4^+$ HOS cells infected with undiluted virus pseudotyped with VI 1249 were 85,000 and 238,477 LU respectively. None of the BCN or non-BCN Envs mediated CD4-independent infection (data not shown).

Viruses pseudotyped with R2 Env and Envs from 6 BCN and 11 non-BCN donors, as described in Table 1, were tested for neutralization by BCN and non-BCN sera previously identified in studies conducted by Beirnaert et al. (2000) and Donners et al. (2002). The BCN sera were collected 6 months after the sample used in the generation of the BCN envelope clones, except in the cases of Envs 24/00/4 and VI 423, for which the sera corresponded to the same times of the PBMC collections. Sera corresponding to the specific non-BCN Env donors used in this study were unavailable. However, the non-BCN sera used in this study were selected from a panel of sera classified based on low-to-absent neutralizing potency against the aforementioned primary isolates CA 4, CA 13 and VI 686 (Donners et al., 2002). As shown in Fig. 1A, the BCN sera neutralized virus pseudotyped with the R2 Env and each of the BCN Envs at titers ranging from 1:8 to 1:2048 (overall geometric mean titer (GMT) of the BCN sera to neutralize BCN Envs = 1:110). The non-BCN sera neutralized the BCN Envs at titers ranging from 1:4 to 1:1024. Viruses pseudotyped with Env 14/00/4 and VI 423 were neutralized significantly more by BCN than non-BCN sera. The geometric mean titers were higher against viruses pseudotypes with R2 Env and BCN Envs 24/00/4, VI 1793, VI 843 and VI 1249 for the BCN than the non-BCN sera, although statistical significance of these differences was not demonstrated. As shown in Fig. 1B, the BCN and non-BCN sera neutralized viruses pseudotyped with non-BCN Envs at titers ranging from 1:8 to 1:1024. Among the non-BCN Envs, virus pseudotyped with Envs VI 1273, MACS#4, CA1 and NYU1423 was neutralized significantly more by BCN than non-BCN sera. Viruses pseudotyped with other non-BCN Envs were also neutralized more by BCN than non-BCN sera, although these differences were not statistically significant. Two non-BCN Envs, notably CA1 and 93BR029, displayed low specificity and were neutralized at titers $\geq$1024 by all the BCN sera and by four non-BCN sera (VI1401, VI 1400, VI 0998 and VI1295). The low specificity of the CA1 virus to neutralization by diverse HIV-1 sera was previously observed (Nyambi et al., 1996).

Small sample sizes may have limited our ability to determine statistically significant differences between neutralization of some pseudotyped viruses by BCN and non-BCN sera. To further test the consistency of trends for greater neutralization by the BCN and non-BCN sera, we compared GMTs for BCN and non-BCN sera using paired Student's *t* test. The overall GMTs of BCN and non-BCN sera against viruses pseudotyped with BCN Envs were 1:110 and 1:38, respectively ($P = 0.006$). The overall GMTs of BCN and non-BCN sera against viruses pseudotyped with non-BCN Envs were 1:115 and 1:42, respectively ($P = 0.0007$). The overall GMTs for neutralization of viruses pseudotyped with the BCN and non-BCN Envs by BCN and non-BCN sera were 1:113 and 1:41, respectively ($P = 5.14 \times 10^{-6}$). The differences in titers of BCN and non-BCN sera remained significant if titers against the homologous pseudotyped viruses were not included in the comparisons ($P = 1.28 \times 10^{-5}$).

Neutralization of viruses pseudotyped with BCN Envs 24/00/4 and VI 423 by contemporaneous serum was observed. This observation could reflect that Env clones were obtained from genomic proviral DNA, instead of plasma viral RNA. For that reason, the Env clones may represent historically relevant envelope glycoproteins, but not Envs present on viruses actually being produced at time of sampling.

Among the BCN sera, the one with the lowest overall GMT (1:42) was VI 1249/8. An earlier serum from this donor was previously classified as having lower levels of cross-reactive neutralizing activity (Beirnaert et al., 2000). Moreover, this donor was infected with a subtype CRF01_AE strain, and cross-reactive neutralization of non-CRF01_AE strains by such sera is expected to be low (Mascola et al., 1994; Zhang et al., 2002).

Comparative neutralization properties of BCN and non-BCN pseudotypes by Mabs and sCD4

To determine whether BCN Envs displayed greater comparative sensitivity to neutralization at particular epitopes than non-BCN Envs, we compared neutralization of viruses pseudotyped with BCN and non-BCN Envs using two-domain sCD4 and a panel of cross-neutralizing Mabs, as illustrated in Fig. 2. Fab 19b, 4KG5, Z13 and 4.8d neutralized the BCN and non-BCN Env-pseudotyped viruses infrequently, and results for those Mabs are not shown. The R2-pseudotyped virus was neutralized

by each of the gp120-specific Mabs, as expected. BCN Envs were of comparable susceptibility to neutralization by sCD4 and the gp120 Mabs X5, IgG1b12, 17b, 2G12 and 447-52d as the non-BCN Envs (Fig. 2). As was the case for serum neutralization, the non-BCN Envs CA1 and 93BR029 were always sensitive to neutralization by these gp120 Mabs. The sensitivity of these two non-BCN Envs to neutralization by both human sera and gp120 Mabs is consistent with the interpretation that these two clones possessed a global neutralization sensitivity phenotype (Donners et al., 2003; Nyambi et al., 1996).

Viruses pseudotyped with Envs from five of the six BCN donors exhibited sensitivity to neutralization by the anti-gp41 Mabs, 2F5 and 4E10. BCN Envs were neutralized by 2F5 at ID_{50} ranging from 0.2 to 3 μg/ml (Fig. 2). The R2 Env was also sensitive to 2F5 neutralization, consistent with a previous report (Zhang et al., 2002). Virus pseudotyped with BCN Env, VI 843, was resistant to neutralization by Mab 2F5 at 50 μg/ml. The Mab 2F5 neutralized viruses pseudotyped with the non-BCN Envs at ID_{50} ranging from 0.39 to 25 μg/ml. Most of the viruses pseudotyped with the BCN Envs were also sensitive to neutralization by Mab 4E10, with ID_{50} ranging from $\leq$0.2–<6.25 μg/ml. Env VI 843, which was resistant to 2F5, displayed intermediate resistance to neutralization by Mab 4E10, with ID_{50} = 12.5 μg/ml. The sensitivity to neutralization by 4E10 of viruses pseudotyped with the non-BCN Envs ranged from 1.56 to 25 μg/ml. One of the globally sensitive non-BCN Env 93BR029 was the most sensitive of the non-BCN Envs to neutralization by the gp41 Mabs, while the other, CA1, displayed intermediate resistance to the gp41 Mabs.

Two BCN Envs that were sensitive to neutralization by Mab 2F5, 14/00/4 and 24/00/4, respectively were derived from samples of two donors with the most potent BCN sera as defined by Beirnaert et al. (2000). These two Envs were resistant to all Mabs targeting gp120 epitopes, and 24/00/4 was also resistant to sCD4 (Fig. 2). Likewise, samples obtained 6 months after the sample that yielded Env clones 14/00/4 and 24/00/4 were used for production of additional Env clones. The sensitivity of viruses pseudotyped with the early and late Envs from these donors to Mabs 2F5 and 4E10 is shown in Figs. 3A and 4A. Of two late clones from donor 14/00, clone 14/00/8-33 was sensitive to neutralization by Mabs 2F5 and 4E10, while 14/00/8-83 was relatively resistant to both Mabs (2F5 ID_{50} = 0.01 vs. >12.5μg/ml, 4E10 ID_{50} = 0.2 vs. 7.8 μg/ml; Fig. 3A). Of three Env clones obtained from the late sample from donor 24/00, clones 24/00/8-46 and 24/00/8-275 were sensitive to neutralization by Mab 2F5, similar to Env 24/00/4, while the late clone 24/00/8-258 was relatively resistant (Fig. 4A). The early and late Env clones from this donor displayed similar sensitivity to Mab 4E10. The finding that late clones from each of these donors were resistant to 2F5 raised the possibility that neutralization escape mutants had emerged in each donor as a result of selection by antibodies in their serum. The variable sensitivity of Env clones from these two donors to neutralization by these Mabs did not result from virus cultivation since early and late Env clones were generated from uncultured PBMC samples (Beaumont et al., 2004; Schuitemaker and Kootstra, 2005).

Amino acid sequence variability within the 2F5 and 4E10 epitopes

We analyzed the amino acid sequences of the region of gp41 that includes the epitopes targeted by Mabs 2F5 and 4E10, as shown in Table 2. The 2F5 epitope has been reported to include the 13 amino acid sequence ELLELDKWASLWN, extending from residue 659 through residue 671 of gp160. In particular, the residues DKW at positions 664–666 have been reported to be critical for Mab 2F5 binding (Barbato et al., 2003; Binley et al., 2004; Li et al., 2005; Ofek et al., 2004; Zwick et al., 2004, 2005). Substitutions within the DKW tripeptide were observed only in the two BCN Env clones, 24/00/8-258 and VI 843, that were resistant to neutralization by Mab 2F5 at 50 μg/ml. These substitutions were K665T in 24/00/8-258 and D664G in VI 843. Two other substitutions, A667D and N671S in VI 843, may have contributed to 2F5 resistance. The only 2F5 epitope substitution that distinguished the Env clones that were highly sensitive to neutralization by Mab 2F5, 14/00/4 and 14/00/8-33, from the Env that was more resistant to neutralization, 14/00/8-83, was A662T. Commonly observed substitutions in other BCN Env that apparently did not contribute to resistance to neutralization by Mab 2F5 included E659D and E662A (Cardoso et al., 2005; Trkola et al., 1995; Zwick et al., 2005). These latter two substitutions were also commonly observed in the non-BCN Envs. Besides these, the only substitutions observed in the 2F5 epitope sequence were E662S and N671S in Env CA1 and S668G in Env NYU1423. Viruses pseudotyped with these two Env were intermediately resistant and resistant to neutralization by Mab 2F5, respectively (ID_{50} of 12.5 and 25 μg/ml) (Fig. 2).

The 4E10 epitope has been defined as the six-amino-acid-peptide NWFDIT, extending from amino acid residue 671 through 676 of gp160. Two common substitutions, D674N and T676S, within the 4E10 epitope did not appear to correlate with neutralization resistance (Binley et al., 2004; Cardoso et al., 2005; Zwick et al., 2001, 2005). The BCN Env clone VI 843, which was intermediately resistant to neutralization by Mab 4E10, had the additional substitution N671S. Other substitutions in the non-BCN Envs that may account for moderate resistance to neutralization by Mab 4E10 were N671S and D674E in Env CA1 and D674S in Env MACS4. No amino acid substitutions were apparent within the 4E10 epitope sequence of Envs NYU1026 and NYU1423 that might account for the relative resistance of those Envs to neutralization by Mab 4E10. No mutation in the 4E10 epitope sequence distinguished the donor 14/00 Env clones that were highly sensitive to neutralization by Mab 4E10, 14/00/4 and 14/00/8-33 (ID_{50} = 0.2 μg/ml) from the clone that was less sensitive, 14/00/8-83 (ID_{50} = 7.8 μg/ml) (Fig 3A).

Contribution of the A662T mutation in the 14/00/8-83 Env to resistance to neutralization by Mabs 2F5 and 4E10

The mutations T662A in 14/00/8-83 and K665T in 24/00/8-258 are of particular interest because of their potential contributions to the moderate and full resistance to neutralization of those clones to Mab 2F5. The 662T sequence in the

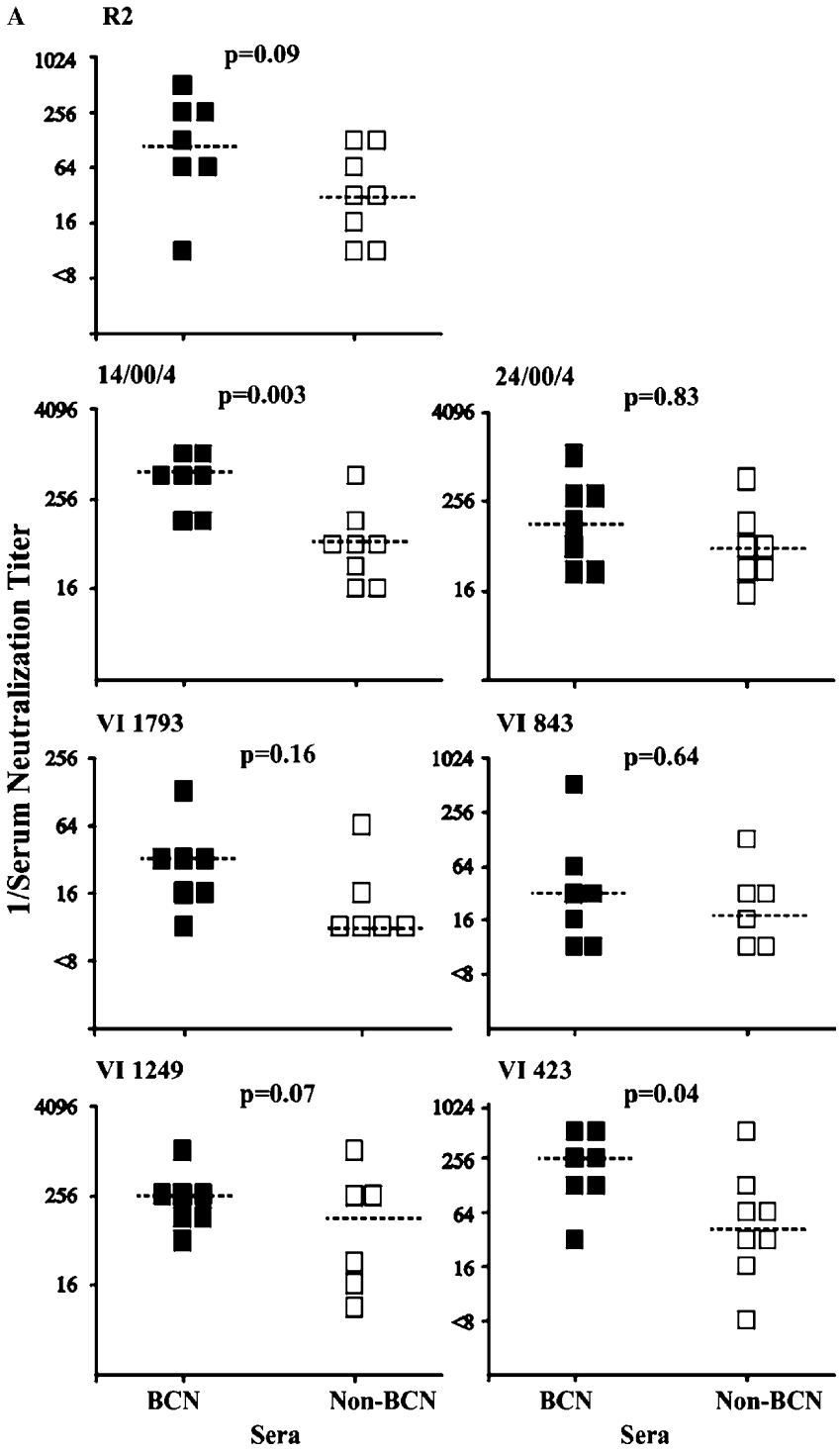


Fig. 1. Comparative neutralization of viruses pseudotyped with Envs of BCN (A) and non-BCN (B) donors by sera from BCN ■ and non-BCN donors □. Assays were performed in triplicate. Results are from single experiments or are averages from two experiments in a few cases. Neutralization titers were defined as the highest serum dilution that resulted in greater than or equal to 50% inhibition of luciferase activity. Due to sample limitation, pseudotyped viruses VI 843, VI 1249, VI 1793, 93BR20.9 and NYU1026 were not tested for neutralization by serum from donor VI 0747. Likewise, serum from donor VI 1071 was not tested against pseudotyped viruses VI 843, 24/00/4, VI 1249, VI 1793, 93BR029, CA1, NYU 1423 and NYU1026. The horizontal dashed lines demonstrate the geometric mean titers of neutralization of each Env by the panel of BCN and non-BCN sera. *P* values on charts are results of two-tailed Student's *t* tests comparing geometric means of BCN and non-BCN serum titers. The overall GMTs for neutralization of viruses pseudotyped with the BCN and non-BCN Envs by BCN and non-BCN sera were 1:113 and 1:41, respectively ($P = 5.14 \times 10^{-6}$ by paired Student's *t* test), comparing the geometric metric means of BCN and non-BCN sera against each virus.

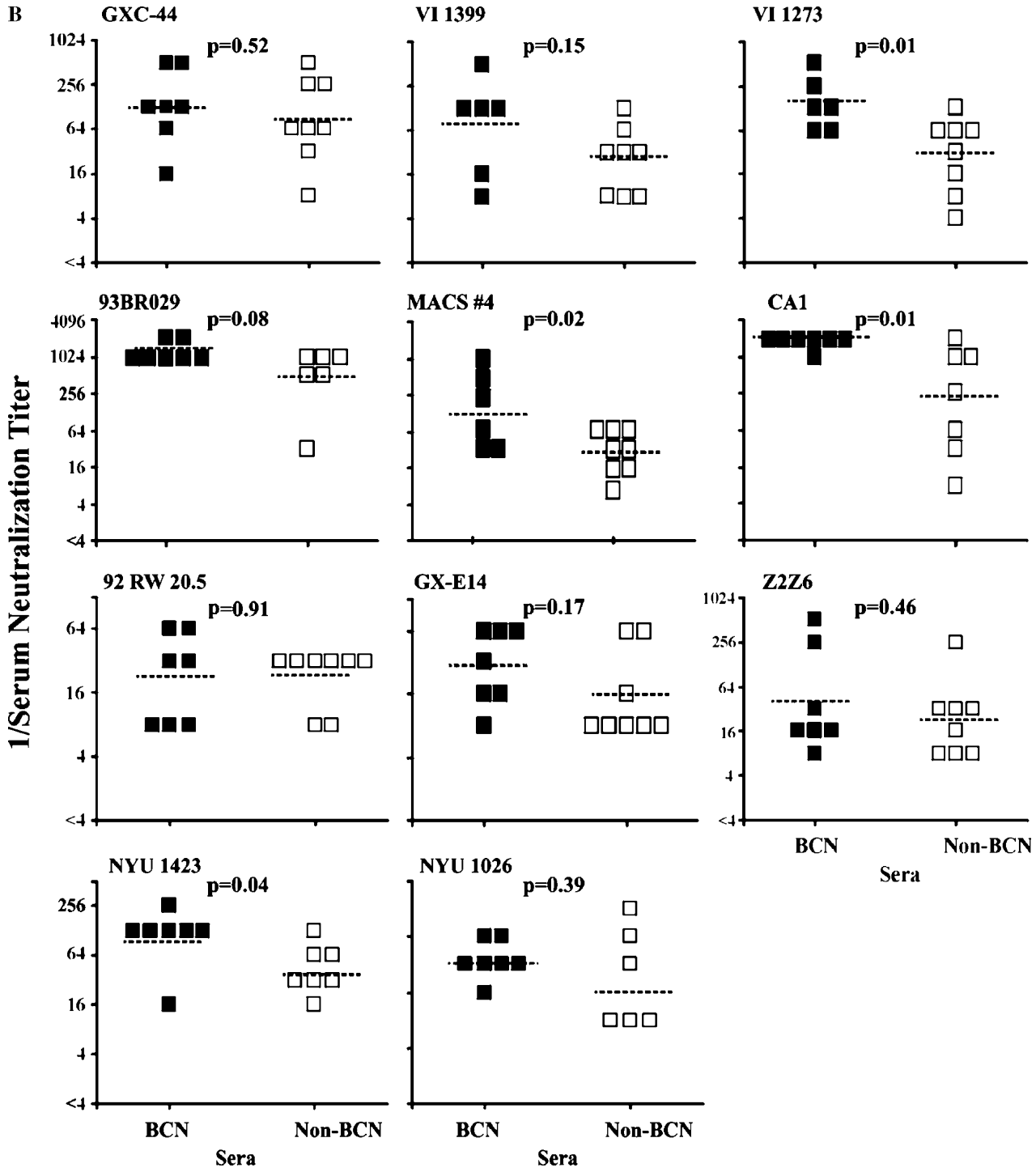


Fig. 1 (*continued*).

Envs 14/00/4 and 14/00/8-33 is very unusual. We found one other sequence with this substitution in the HIV and GenBank databases (HIV-1 ARMA037; accession # AY037277) (Carr et al., 2001). To investigate whether this unusual mutation confers susceptibility to Mab 2F5 neutralization, we used site-directed mutagenesis to introduce the T662A mutation into clone 14/00/4 and to introduce the reverse mutation (A662T) into the non-BCN clones, NYU1026 and NYU1423, which were sensitive and resistant to Mab 2F5 neutralization, respectively. The effects of these mutations on sensitivity to neutralization by Mabs 2F5 and 4E10 are shown in Fig. 3B. The alanine substitution into Env 14/00/4 changed it from highly sensitive to relatively resistant to neutralization by Mabs 2F5 (ID_{50} = 0.45 vs. 6.25 μg/ml) and 4E10 (ID_{50} = 0.9 vs. 9.34 μg/ml). Introduction of threonine at the same position of Env NYU1026 had the reverse effect on sensitivity to neutralization by Mabs 2F5 (ID_{50} = 3.13 vs. 0.31 μg/ml) and 4E10 (ID_{50} = 10.41 vs. 0.78 μg/ml). The magnitude of the effects of these

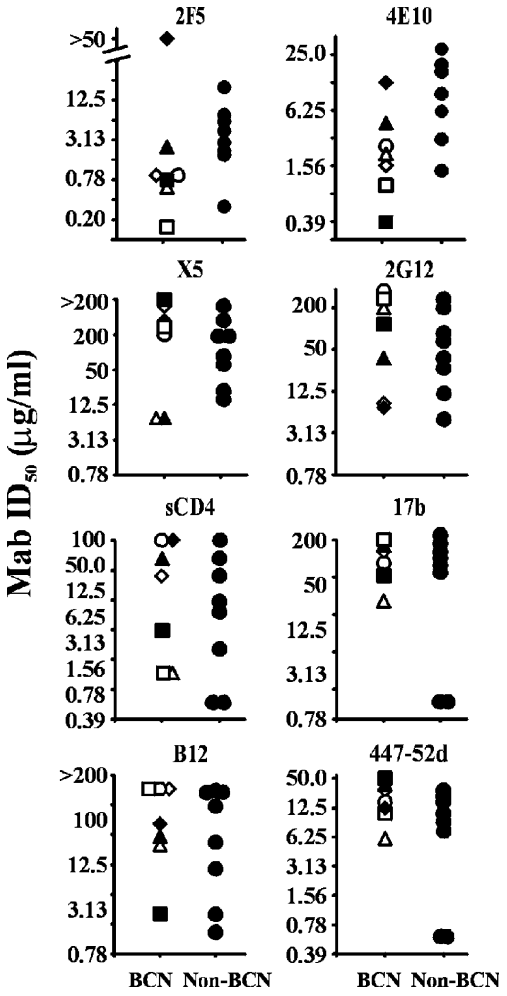


Fig. 2. Neutralization of viruses pseudotyped with BCN and non-BCN Envs by Mabs and sCD4. Results are shown for viruses pseudotyped with the BCN and non-BCN Envs as follows: R2: △ 14/00/4: □; 24/00/4: ○ VI 423: ▲; VI 843: ♦; VI 1249: ■; and VI 1793: ◇; all non-BCN Env are shown as ●. Neutralization assays were performed in triplicate, and results shown are geometric means of two independent experiments. Mabs were tested for neutralization in serial two-fold dilutions. The 50% inhibitory dose (ID_{50}) was defined as the lowest Mab concentration that resulted in greater than or equal to 50% inhibition of viral infectivity.

substitutions in Envs 14/00/4 and NYU1026 is similar to the relative differences in sensitivity to neutralization by Mabs 2F5 and 4E10 of the Env clones 14/00/4 and 14/00/8-33 compared to 14/00/8-83. Introduction of threonine at the same position of Env NYU1423 caused a small but consistent increase in sensitivity to neutralization by Mab 2F5 (ID_{50} = 17.7 vs. 10.4 μg/ml) and no significant change in sensitivity to neutralization by Mab 4E10. The results demonstrated that the presence of threonine at residue 662 is associated with increased sensitivity to neutralization by both of these Mabs to an extent that depends on the particular Env evaluated.

Thr 662 significantly contributes to the broad cross-neutralizing activity of BCN serum from donor 14/00

To further test the possible relationship of Thr 662 to induction of antibodies against the MPER of gp41, we compared sensitivity of virus pseudotyped with Envs 14/00/4 and 14/00/4 T662A mutant to neutralization by BCN (14/00/8, 24/00/8, HNS2) and non-BCN (VI 1077, VI 1295, VI 1400) sera. As illustrated in Fig. 3C, the T662A mutation resulted in 211 and 27-fold resistance to neutralization by serum 14/00/8 and 24/00/8, respectively. In comparison, the mutation had a lesser effect on neutralization by HNS2 serum and the non-BCN sera VI 1295, VI 1400 and VI 1077, with relative resistance of the mutant ranging from 1- to 10-fold. Thus, reduced sensitivity of the 14/00/4 T662A mutant to neutralization by the BCN sera 14/00/8 and 24/00/8, but not by the non-BCN sera, supports the possibility that these particular BCN sera may have relatively high neutralizing activity directed against the MPER of gp41.

Contribution of the K665T mutation in the 24/00/8-258 Env to resistance to neutralization by Mab 2F5

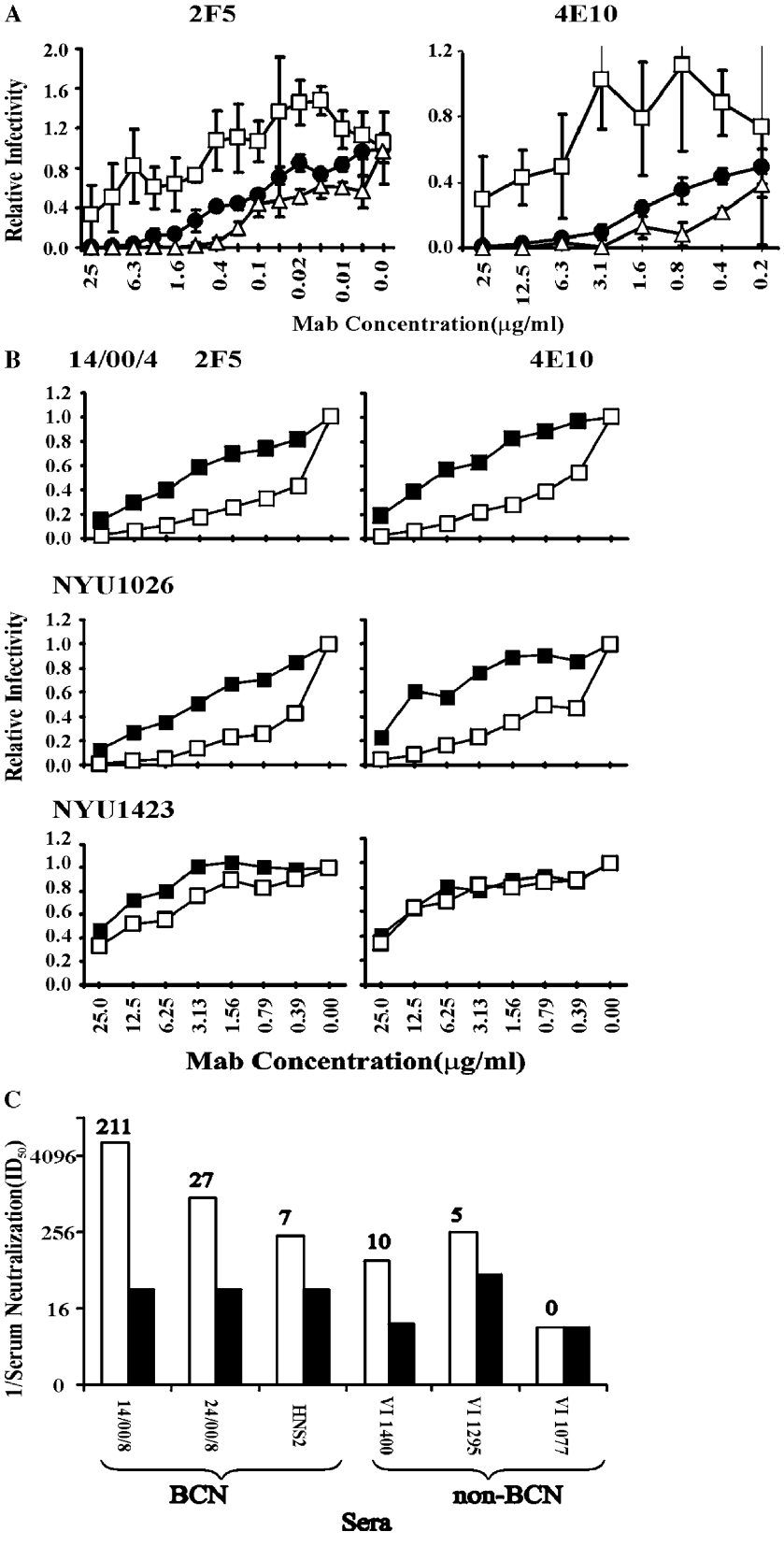
Previous studies have reported that a K665N mutation results in poor binding and resistance to 2F5 neutralization of HIV-1 primary isolates (Conley et al., 1994b; Stiegler et al., 2001). Of three late envelope clones derived from donor 24/00, one (24/00/8-258) was resistant to neutralization by Mabs 2F5 (Fig. 4A) and displayed a single mutation within the 2F5 epitope sequence, K665T. To confirm the relevance of this mutation to neutralization by 2F5, we introduced the K665T point mutation into Env 24/00/4. This mutation caused resistance to 2F5 but had no effect on 4E10 sensitivity (Fig. 4B).

Quasispecies variations in the 2F5 and 4E10 epitopes of BCN donors 14/00 and 24/00

To further evaluate whether the late Envs 14/00/8-83 and 24/00/8-258, which were relatively resistant to neutralization by Mab 2F5, represented emergence of neutralization-resistant escape variants in these donors, we examined quasispecies variation at the 2F5 and 4E10 epitopes in each of these donors. For this purpose, using uncultured PBMC genomic DNA as template for PCR, we cloned and analyzed amino acid sequences of the MPER of gp41 from early and late PBMC samples from these two donors. Results of these analyses are shown in Tables 3 and 4. In donor 14/00, 9 of 10 early and all the late gp41 clones

Fig. 3. Effects of Thr 662 on sensitivity to neutralization by gp41 Mabs and polyclonal serum. (A) Variable sensitivity of viruses pseudotyped with early 14/00/4 (●) and late 14/00/8-33 (△) and 14/00/8-83 (□), Env clones from donors 14/00 to neutralization by Mabs 2F5 and 4E10. Relative infectivity is the ratio of luciferase units obtained in the presence of Mab compared to medium. (B) Comparative effects of T662 and A662 on sensitivity to neutralization by the Mabs 2F5 and 4E10. Viruses pseudotyped with the 14/00/4, NYU1026 and NYU1423 Envs were compared for neutralization by Mabs 2F5 and 4E10. Site-directed mutagenesis was used to construct the 14/00/4 (A662), NYU1026 (T662) and NYU1423 (T662) mutant Envs. Viruses pseudotyped with the Envs having A662 are shown as ■, and viruses pseudotyped with Envs having T662 are shown as □. (C) Comparative neutralization of virus pseudotyped with 14/00/4 (T662) (□) and 14/00/4 (A662) (■) Envs by BCN and non-BCN polyclonal serum. The ID_{50} for each serum was determined by linear regression using Excel. Numbers above each bar are the differences in ID_{50} of virus pseudotyped with 14/00/4 (T662) and 14/00/4 (A662) by each polyclonal serum.

were found to have the neutralization-sensitive 662T sequence (Table 3). However, in donor 24/00, 8 of 11 early, but only 3 of 10 late gp41 clones, were found to have the neutralization-sensitive 665 K sequence (Table 4). These results indicate that neutralization-resistant variants represented by clone 24/00/8-258 had emerged as the dominant populations in this donor, consistent with the emergence of neutralization escape mutants. However, absence of the 662A sequence in clones from donor



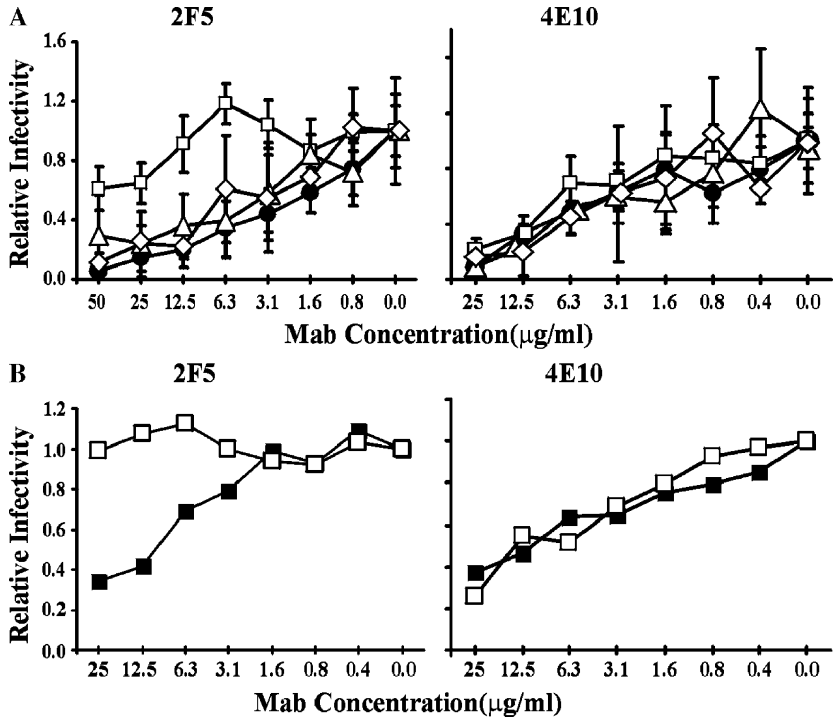


Fig. 4. Effects of the K665T mutation in Env clones of donor 2400 to neutralization by Mabs 2F5 and 4E10. (A) Variable sensitivity of early 24/00/4 (●) and late 24/00/8-46 (△), 24/00/8-275 (◇) and 24/00/8-258 (□) Envs clones from donors 24/00 to neutralization by Mabs 2F5 and 4E10. Viruses pseudotyped with these Envs were tested for neutralization by the two Mabs. Each result shown is from one experiment and is essentially the same as those from two replicate experiments. All experiments were performed in triplicate. (B) The K665T mutation in Env 24/00/8 determines resistance to neutralization by Mab 2F5. Viruses pseudotyped with the 24/00/4 (K665) (■) and 24/00/4 (T665) (□) Env were tested for neutralization by the Mabs 2F5 and 4E10. Assays were carried out in triplicate, and results shown are averages of two independent experiments.

14/00 does not negate the possibility that the sequence may have emerged through escape mutation. Additional evidence of escape might have been observed if analyses had included sequences of plasma viral RNA or later samples from the donor.

Discussion

Neutralizing antibodies are commonly required for vaccine-induced protection against viral infections and are likely

Table 2
Amino acid sequences of the gp41 regions comprising the 2F5 and 4E10 epitopes of functional env clones from BCN and non-BCN donors

Donors		Amino acid sequence[a]	Neutralization by Mab[b]	
Type	Number		2F5	4E10
BCN	VI 423	ELLELDKWASLWNWFDIT	S	S
	VI 843	ELLELGKWDSLWSWFDIS	R	I
	14/00/4	ELLTLDKWASLWNWFDIS	S	S
	14/00/8-83	ELLALDKWASLWNWFDIS	I	I
	VI 1249	DLLELDKWASLWNWFDIT	S	S
	24/00/4	DLLALDKWASLWNWFNIS	S	S
	24/00/8-258	DLLALDTWASLWNWFNIS	R	R
	VI 1793	DLLALDKWASLWNWFDIS	S	S
Non-BCN	NYU1423	DLLALDKWAGLWNWFDIS	R	R
	VI 1273	ELLELDKWASLWNWFDIT	I	I
	VI 1399	ELLELDKWASLWNWFDIT	S	S
	MACS4	ELLELDKWASLWNWFSIT	S	I
	93BR029[c]	ELLALDKWASLWNWFDIS	S	S
	GXE-14[c]	DLLELDKWASLWNWFDIT	S	R
	NYU1026	DLLALDKWASLWNWFDIT	S	R
	CA1[c]	ELLSLDKWASLWSWFEIS	I	I

[a] The 2F5 epitope sequence, as described by Barbato et al. (2003), is shown as homologous to the 13-mer ELLELDKWASLWN (Barbato et al., 2003). The 4E10 epitope sequence, as described by Zwick et al. (2001), is shown as homologous to the six-mer NWFDIT (Zwick et al., 2001). Residues differing from those sequences are decorated with shaded boxes.

[b] For 2F5, strains were considered to be sensitive (S), intermediate (I) or resistant (R) if their ID_{50} were ≤3.13, >3.13–12.5 or ≥25 μg/ml, respectively. For 4E10, strains were considered to be sensitive (S), intermediately resistant (I) or resistant (R) if the ID_{50} were ≤6.25, >6.25–12.5, or >12.5 μg/ml, respectively.

[c] gp160 amino acids sequences retrieved from the HIV database (http://www.hiv.lanl.gov).

Table 3
Quasispecies variations in the 2F5 and 4E10 epitopes of the early and late *env* clones from BCN donor 14/00[a]

Clone type	Clones from early sample, 14/00/4		Clones from late sample, 14/00/8	
	Clone number	2F5/4E10 amino acid sequence	Clone number	2F5/4E10 amino acid sequence
gp160 full length	14/00/4	ELLTLDKWASLWNWFDIS	14/00/8-33	ELLTLDKWASLWNWFDIS
			14/00/8-83	ELLALDKWASLWNWFDIS
gp140 segment	14/00/4-3	ELLTLDKWAGLWNWFDIS	14/00/8-1	ELLTLDKWASLWNWFDIS
	14/00/4-4	ELLALDKWASLWNWFDIS	14/00/8-2	ELLTLDKWASLWNWFDIS
	14/00/4-5	ELLTLDKWASLWNWFDIS	14/00/8-3	ELLTLDKWASLWNWFDIS
	14/00/4-6	ELLTLDKWASLWNWFDIS	14/00/8-4	ELLTLDKWASLWNWFDIS
	14/00/4-7	ELLTLDKWASLWNWFDIS	14/00/8-5	ELLTLDKWASLWNWFDIS
	14/00/4-8	ELLTLDKWASLWNWFDIS	14/00/8-6	ELLTLDKWASLWNWFDIS
	14/00/4-10	ELLTLDKWASLWNWFDIS	14/00/8-8	ELLTLDKWASLWNWFDIS
	14/00/4-11	ELLTLDKWASLWNWFDIS	14/00/8-9	ELLTLDKWASLWNWFDIS
	14/00/4-12	ELLTLDKWASLWNWFDIS	14/00/8-10	ELLTLDKWASLWNWFDIS
	14/00/4-15	ELLTLDKWASLWNWFDIS	14/00/8-11	ELLTLDKWASLWNWFDIS
			14/00/8-12	ELLTLDKWASLWNWFDIS
			14/00/8-13	ELLTLDKWASLWNWFDIS
			14/00/8-14	ELLTLDKWASLWNWFDIS

[a] Amino acid sequences corresponding to the epitope regions defined in Table 2 inferred from nucleotide sequences of cloned gp41 coding segments. Decorations indicate residues that vary from the sequence of the full-length 14/00/4 Env clone. Clones 14/00/4, 14/00/8-33 and 14/00/8-83 are functional, full-length Env clones; the remaining clones consist of gp41 sequences only. GenBank accession numbers for clones 14/00/8-33 and 14/00/8-83 are DQ313240 and DQ313241, respectively.

to be important for vaccine-induced protection against HIV-1 infection (Burton et al., 2004a, 2004b; Li et al., 2005; Mascola et al., 2005; Moore and Burton, 2004; Quinnan, 1997). Occasionally, patients develop neutralizing antibody responses that are highly cross-reactive. In this study, we tested the possibility that Envs from such donors possess neutralization epitope properties that are related to their BCN antibody responses (Beirnaert et al., 2000, 2001; Donners et al., 2002; Nyambi et al., 1996; Quinnan et al., 1999, 2005; Zhang et al., 1999, 2002).

The neutralization-sensitive, CD4-independent R2 Env derived from the donor of the Human Neutralizing Serum 2 (HNS2) was a paradigm in our study (Quinnan et al., 1999; Vujcic and Quinnan, 1995; Zhang et al., 2002). The capacity of HIV-1 to mediate CD4-independent infection maybe associated with enhanced capacity to induce neutralizing antibodies (Dong et al., 2003; Edwards et al., 2001; Endres et al., 1996; Hoxie et al., 1998; Quinnan et al., 2005; Zwick et al., 2003). However, the BCN Envs obtained in the present study could not mediate CD4-independent infection, causing us to examine whether other conserved Env characteristics may have been relevant to the induction of BCN antibodies in the sera from these donors.

We conducted studies to examine the neutralizing activity of sera from the BCN donors previously identified in ITM studies. These previous studies were conducted using replicating primary viruses and peripheral blood mononuclear cells (PBMC) as indicator cells. Their results demonstrated

Table 4
Quasispecies variations in the 2F5 and 4E10 epitopes of the early and late *env* clones from BCN donor 24/00[a]

Clone type	Clones from early sample, 24/00/4		Clones from late sample, 24/00/8	
	Clone number	2F5/4E10 amino acid sequence	Clone number	2F5/4E10 amino acid sequence
gp160 functional	24/00/4	DLLALDKWASLWNWFNIS	24/00/8-46	DLLALDKWASLWNWFNIS
			24/00/8-275	DLLALDKWASLWNWFNIS
			24/00/8-258	DLLALDTWASLWNWFNIS
gp41 segment	24/00/4-1	DLLALDKWASLWNWFDIS	24/00/8-1	DLLALDTWASLWNWFNIS
	24/00/4-2	DLLALDKWESLWNWFNIS	24/00/8-3	DLLALDKWAGLWNWFDIT
	24/00/4-5	DLLALDTWASLWNWFNIS	24/00/8-4	DLLALDTWASLWNWFNIS
	24/00/4-7	DLLALDTWASLWNWFNIS	24/00/8-6	DLLALDTWASLWNWFNIS
	24/00/4-8	DLLALDKWESLWNWFNIS	24/00/8-7	DLLALDKWENLWNWFNIS
	24/00/4-9	DLLALDKWASLWNWFNIT	24/00/8-8	DLLALDKWENLWNWFNIS
	24/00/4-10	DLLALDKWASLWNWFDIS	24/00/8-10	DLLALDTWASLWNWFNIS
	24/00/4-12	DLLALDKWASLWNWFDIS	24/00/8-11	DLLALDTWASLWNWFNIS
	24/00/4-13	DLLALDTWASLWNWFNIS	24/00/8-13	DLLALDTWASLWNWFNIS
	24/00/4-14	DLLALDKWASLWNWFDIS	24/00/8-16	DLLALDTWASLWNWFNIS
	24/00/4-15	DLLALDKWASLWNWFNIT		

[a] Amino acid sequences corresponding to the epitope regions defined in Table 2 inferred from nucleotide sequences of cloned gp41 coding segments. Decorations indicate residues that vary from the sequence of the full-length 24/00/4 Env clone. GenBank accession numbers for clones 24/00/8-46, 24/00/8-275 and 24/00/8-258 are DQ313243, DQ313245 and DQ313244, respectively.

quite remarkable differences between sera from BCN and non-BCN donors. In the present study, assays were conducted using pseudotyped viruses and Human Osteosarcoma (HOS) cells as indicator cells. Pseudotyped virus assays in cell lines tend to be more sensitive for detection of low serum neutralizing activity than PBMC-based assays. This difference may account for greater well-to-well precision of the pseudotype virus assays versus assays that depend upon multiple cycles of replication of primary viruses. Additionally, cloned viruses may be more sensitive to neutralization than parental uncloned viruses (Binley et al., 2004; Dong et al., 2003; Li et al., 2005; Mascola et al., 2005; Quinnan et al., 1998; Zhang et al., 1999). Most importantly, we did not have available pseudotype viruses representative of all the viruses used in the ITM studies, particularly the group O virus. Notwithstanding, the BCN sera neutralized all the primary Env-pseudotyped viruses, while non-BCN sera all failed to neutralize one or more strains tested. The overall geometric mean titers of the BCN sera were also higher than those of the non-BCN sera. Thus, our serum neutralization results generally confirmed those reported previously (Beirnaert et al., 2000; Donners et al., 2002).

Our pursuit of the possibility that characteristics of the MPER of gp41 may distinguish BCN from non-BCN Envs was based initially on observation that the BCN and non-BCN Envs were not distinguished by sensitivities to anti-gp120 Mabs. Rather, the majority of BCN Envs were neutralized at lower concentrations of 2F5 and 4E10 than the non-BCN Envs and than commonly reported for primary strains of HIV-1 (Binley et al., 2004; Cardoso et al., 2005; Conley et al., 1994b; Li et al., 2005; Muster et al., 1994; Parren et al., 1998; Stiegler et al., 2001; Trkola et al., 1995, 1998). Two of the BCN donors 14/00 and 24/00 were selected for further study of this issue, based on their having the most cross-reactive serum neutralizing activities among the group as reported by Beirnaert et al. and their Envs being among the most sensitive to neutralization by 2F5 and 4E10.

Env clones obtained from later time point samples of donor 14/00 and 24/00 showed evidence of neutralization escape mutation. In both cases, late clones were obtained that were significantly more resistant to neutralization by 2F5 than the original Env clones. Sequence analyses of these clones revealed 2F5 epitope mutations potentially responsible for the resistance, and site-directed mutagenesis of homologous and heterologous Envs confirmed the roles of the mutations in determining resistance to 2F5. Of note, the 2F5 epitope mutation in Env from donor 14/00 also conferred resistance to 4E10. Quasispecies analyses confirmed the emergence of the 2F5-resistant genotype in donor 24/00, but not donor 14/00. However, the 2F5 resistance mutation in Env clone from donor 14/00 conferred resistance to neutralization by sera from donor 14/00, providing support for the possibility that it constituted a neutralization escape mutation. It is possible that analyses of quasispecies variation in plasma RNA or in later PBMC samples of donor 14/00 would reveal quasispecies variations typical of escape mutation. We have not yet conducted studies to determine whether quasispecies variation is more consistent overall with 2F5 epitope escape mutation in our BCN donors than in non-BCN donors. However, the evidence reported here is consistent with that possibility.

Structural studies of synthetic oligopeptides bound by each of these Mabs have been published (Barbato et al., 2003; Cardoso et al., 2005; Ofek et al., 2004; Zwick et al., 2004). However, attempts to induce neutralizing antibodies against these epitopes using synthetic oligopeptides as immunogens have been unsuccessful. The failure of induction of neutralizing antibodies by synthetic peptide immunization suggests that the sequence is constrained in the intact Env complex in a conformation that is relevant to induction of antibody responses. Neutralizing Mabs that target the MPER of gp41 are rare in infected individuals (Cardoso et al., 2005; Kang et al., 2005; Nabel, 2005; Ofek et al., 2004; Rusert et al., 2005; Zwick et al., 2001, 2004, 2005). Haynes et al. have noted that the structure of HIV-1 neutralizing antibodies, all of which have extended CDRH3 regions, is typical of auto antibodies and that these Mabs cross-react with human antigens targeted by autoimmune responses (Haynes et al., 2005; Nabel, 2005). This cross-reactivity may explain the rarity of broadly cross-reactive human Mabs against HIV-1. Our evidence indicating that BCN donors 14/00 and 24/00 may have developed neutralization escape mutation in this region is, therefore, of interest. Moreover, the observations that the rare 662T sequence in Env of donor 14/00 was specifically associated with very high sensitivity to neutralization by Mabs 2F5 and 4E10, but not other Mabs, and to neutralization by homologous sera constitute additional evidence of the structural complexity of this region of gp41 and of the possibility that this Env may be exceptionally competent for induction of anti-MPER neutralizing antibodies.

The strategy used to identify BCN donors is likely related to the epitope specificity of the BCN antibodies in their sera. In particular, the inclusion of a Group O isolate in the panel of key isolates used by the ITM studies probably resulted in identification of donors with antibodies directed against the MPER of gp41 (Beirnaert et al., 2000; Donners et al., 2002). Moreover, group O viruses are sensitive to neutralization by Mabs 2F5 and 4E10, but not other human Mabs (Beirnaert et al., 2001; Ferrantelli et al., 2004; Trkola et al., 1995). Additional studies to evaluate the potential immunogenicity of the MPER of gp41 in the 14/00/4 and 24/00/4 Envs should include more extensive evaluation of quasispecies variation in the donors at sequential time points, characterization of the epitope specificity of neutralizing antibodies in their sera and evaluation of the immunogenicity of the Envs.

Materials and methods

Human sera

Sera collected from six BCN and eight non-BCN donors previously characterized in clinical cohort studies at the AIDS Reference Center of the Institute of Tropical Medicine (ITM) in Antwerp, Belgium were used in the present study (Beirnaert et al., 2000; Donners et al., 2002). Five of the BCN sera, VI 423, 14/00/8, VI 843, 24/00/4 and VI 1793, were collected from

donors whose sera neutralized the three key primary viruses (MN, VI 525 and CA 9) with high potency (set 1 of Beirnaert et al.) and one (set 2: VI 1249/8) neutralized with moderate potency. These sera were collected from 4 to 17 months after those described by Beirnaert et al., except serum 14/00/8 which was collected approximately 1 year before the previously published serum. The serum of BCN donors 14/00 and 24/00 was the most potent of the set 1 sera in the Beirnaert study (Beirnaert et al., 2000). The eight non-BCN sera used (VI 0744, VI 0747, VI 0998, VI 1071, VI 1077, VI 1295, VI 1400 and VI 1401) previously exhibited weak or absent neutralization of the primary viruses CA 4, CA 13 and VI 686 (Donners et al., 2002). Due to sample limitation, the non-BCN serum VI 1071 was not tested against four BCN Envs (VI 843, 24/00/4, VI 1249 and VI 1793) and four non-BCN Envs (93BR029, CA1, NYU 1423 and NYU1026). The reference reagent, HNS2, was obtained from National Institute of Health AIDS Research and Reference Reagent Program (ARRRP), as contributed by Luba Vujcic and Gerald Quinnan. Sera were heated at 56 °C for 30 min prior to use and stored at −20 °C.

Cloning of HIV-1 envelope genes

Cryopreserved PBMC were the source of HIV-1 proviral DNA of six antiretroviral-therapy-naive HIV-1 BCN donors (Table 1) and two non-BCN donors (VI 1399 and VI 1273). Two separate PBMC samples from donor 14/00 and 24/00, which had been obtained at 6-month intervals, were used as sources of *env* genes. DNA extracts from co-cultured PBMC of four HIV-1-infected donors participating in ongoing cohort studies at the New York Veterans Administration Medical Center, who had not been selected based on neutralizing antibody status, were included as sources of non-BCN *env*. These Env designations were CA1, NYU1026, NYU1423 and 93BR029 (Nadas et al., 2004; Nyambi et al., 1996; Zhong et al., 2002, 2003; Zolla-Pazner et al., 2004). The Env clones MACS#4; GXC-44, GXE-14 and Z2Z6 have been described previously (Quinnan et al., 1998; Zhang et al., 1999). The plasmid $pSV3_{92RW20.5}$ was obtained from ARRRP, as contributed by Feng Gao and Beatrice Hahn (Gao et al., 1996). The previously cloned and characterized R2 Env clone was also used (Quinnan et al., 1999; Zhang et al., 2002). Genetic subtype, geographic origin, year of sample collection and luminescence infectivity reading of virus pseudotyped with each Env are shown in Table 1.

Envelope genes were synthesized by nested polymerase chain reaction (PCR) using the high fidelity rTth DNA polymerase (Applied Biosystems, Foster City, CA). For template, genomic DNA was extracted using the Qiagen Blood and Tissue Kit (Qiagen Inc., Valencia, CA) from uncultured or co-cultured PBMC from all donors. Primers used for the first round PCR were based on either subtype B or on Group M consensus sequences. The second round primers were based on subtype B sequence. All primers used in the second round PCR incorporated restriction enzyme recognition sites for use in cloning. The primers used in the nested PCR were as follows: first round primers: subtype B consensus sense (nt 5831–5883; primer location is according to HXB2 numbering): 5′-ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAG-CATCCAGGAAGTCAGCC-3′ and antisense (nt 9035–8994): 5′-GTCATTGGTCTTAAAGGTACCTGAGGTCTGTCTG-GAAAACCC-3′ or group M consensus sense (nt 5954–5983): 5′-GGCTTAGGCATCTCCTATGGCAGGAAGAAG-3′ and antisense (nt 9088-9059): 5′-CCAGTCCCCCCTTTTCT-TTTAAAAAGTGGC-3′; second round primers: sense (nt 5950–5986): 5′-AAAAGGCTTAGGCATCTCCTATGGCAG-GAAGAAGCGG-3′ and antisense (nt 8902–8868): 5′-CTCGAGATACTGCTCCCACCCCATCTGCTGCTGGC-3′. PCR products were visualized on a 0.7% agarose gel and column-purified prior to restriction enzyme digestion and cloning into the vector pSV7d (Quinnan et al., 1998). Env genes of plasmids with correctly sized inserts were screened for function, as described below.

Cell cultures

The Human Osteosarcoma (HOS) cell lines constitutively expressing CD4 and coreceptors for HIV-1, CCR5 or CXCR4, or coreceptors only, were obtained from the ARRRP, as contributed by Nathaniel Landau. HOS cells were maintained in Dulbecco's minimal essential medium (DMEM) (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum, L-glutamine and penicillin–streptomycin (GIBCO), Tylosin (Sigma) and puromycin for maintenance of plasmid stability. The 293T human embryonic kidney cell lines obtained from the American Type Culture Collection (ATCC, Rockville MD; catalog no. 11268) were maintained in Dulbecco's minimal essential medium (GIBCO) supplemented with 10% fetal bovine serum, L-glutamine and penicillin–streptomycin (GIBCO).

Functional assays of HIV-1 envelope glycoprotein

The ability of cloned Envs to mediate infection of HOS $CD4^+CCR5^+$ or HOS $CD4^+CXCR4^+$ cells was assessed using pseudotyped viruses, as previously described (Quinnan et al., 1998). Pseudotyped viruses were produced by calcium phosphate/HEPES buffer co-transfection (Promega, Madison WI) of 293T cells with pNL4-3.luc.E-R (obtained from the ARRRP as contributed by Nathaniel Landau)- and pSV7d-env plasmid. Eighteen hours after transfection, the media were replaced with media supplemented with 0.1 mM sodium butyrate (Sigma), and cells were propagated for an additional 24 h. Supernatant was clarified by centrifugation at 16,000 rpm for 5 min at 4 °C, sterilized by passage through 0.45 μm pore size filters (Millipore, Bedford, MA) and used in infectivity assays (Zhang et al., 1999, 2002). Briefly, two-fold serial dilutions of pseudotyped virus preparation supernatant were added to $1–2 \times 10^4$ HOS $CD4^+CCR5^+$ or $CXCR4^+$ cells per well in microtiter wells. The plates were incubated at 37 °C in 5% CO_2 for 3 days then washed with phosphate-buffered saline and lysed for 30 min with 15 μl of Luciferase Assay System cell lysis buffer (Promega, Madison WI). Luciferase activity was read using a MicroLumat *Plus* luminometer (Wallac, Gaithersburg MD). An individual well was considered positive if the

luciferase activity was at least 10-fold greater than that of the negative control.

Sequencing and analyses of envelope gene clones

Sequencing was performed using a panel of forward and reverse primers designed based on consensus HIV-1 sequences in the Los Alamos National Laboratory HIV sequence database (http://www.hiv.lanl.gov). Products of the sequencing reactions were purified using the Performa DTR gel filtration cartridge (Edge BioSystems, Gaithersburg, MD). Nucleotide sequencing was performed using the di-deoxy cycle sequencing technique on an Applied Systems Model 3100 Genetic Analyzer (Foster City, CA). Sequence alignment was performed using the DNA Star software package, according to the methods of Higgins and Sharp (1988). Individual bases were assigned based on three or more sequences, including sequences in the forward and reverse directions. Uniqueness of sequences was assessed using the National Center for Biotechnology Information (NCBI) website (http://www.nbi.nlm.nih.gov) and the Los Alamos National Laboratory HIV databases (http://www.hiv.lanl.gov). Amino acid residue numbers are assigned throughout this report on the basis of the sequence of the clone HXB2 (GenBank accession number K03455).

Monoclonal antibodies

To characterize the neutralization phenotypes of the envelope used in this study, we used a panel of 11 broadly cross-reactive human monoclonal antibodies (Mabs) and two-domain sCD4. The Mabs used in this study were b12, Z13 and 4KG5, kindly provided by M.B. Zwick and D.R. Burton (Burton et al., 1994; Zwick et al., 2001, 2003); 447-52d kindly provided by S. Zolla-Pazner (Conley et al., 1994a; Kessler et al., 2003; Sharon et al., 2003; Sharpe et al., 2004); X5 kindly provided by D. Dimitrov (Darbha et al., 2004; Labrijn et al., 2003; Moulard et al., 2002); two-domain sCD4 was a gift from Dr. C Broder (USUHS, Bethesda, MD) (Garlick et al., 1990); 2F5, 4E10, 2G12, 17b, 19b and 48d (Calarese et al., 2003; Cardoso et al., 2005; Muster et al., 1993; Ofek et al., 2004; Purtscher et al., 1994; Sanders et al., 2002; Scanlan et al., 2002, 2003; Zwick et al., 2001; Moore et al., 1994, 1995; Wyatt et al., 1995).

Neutralization assays

In this study, we used a pseudotyped reporter virus neutralization assay, as described previously (Dong et al., 2003; Quinnan et al., 2005; Zhang et al., 1999, 2002, 2004). Briefly, neutralization assays were carried out in triplicate by preincubation of 25 μl of two-fold serial dilutions of Mabs, Fabs, sCD4 or polyclonal sera with 25 μl pseudovirus suspension for 1 h at 4 °C. The virus–antibody mixtures were then combined with 150 μl suspensions of $1–2 \times 10^4$ HOS $CD4^+CCR5^+$ or HOS $CD4^+CXCR4^+$ cells in wells of 96-well, white-walled, flat-bottomed tissue culture plates (Costar, Corning NY). The plates were incubated at 37 °C in 5% CO_2 for 3 days then washed with phosphate-buffered saline and lysed for 30 min with 15 μl of Luciferase Assay System cell lysis buffer (Promega, Madison WI). Luciferase activity was read using a MicroLumat Plus luminometer (Wallac, Gaithersburg MD). Neutralization titers were determined based on luminescence, and the endpoint was the last dilution of sera or other ligand at which the mean results from the test samples were less than 50% of the non-neutralized control mean. The concentrations of sera or Mab that resulted in 90% neutralization were always two- to eight- and usually four-fold greater than those that produced 50% neutralization. Neutralization assays for each Env clone were carried out with each Mab in at least two independent experiments. However, due to limited serum volumes, serum neutralization assays were only done once in most cases and twice in a few cases.

Construction of gp41 clones

To study the quasispecies variation within the 2F5/4E10 epitope regions, we used PBMC genomic DNA of BCN donors 14/00 and 24/00 to synthesize a 1.3 kb gp41 fragment. The outer primers previously mentioned were used in the PCR, and the inner primers were: sense (nt 7405–7426): 5′-GGACAGTT-TAATACCACAGGATCC-3′ (designed for 14/00) or 5′-CTGGGAGTACAATAGCACTTGGGG-3′ (designed for 24/00) and antisense (nt 8795–8771): 5′-TTATAGCAAAGCC-CTTTCTAAGCCC-3′.

The PCR products were gel purified and cloned into the TOPO cloning vector (Invitrogen, Carlsbad, CA). We selected 10–16 gp41 clones from 14/00 and 24/00 and analyzed by sequencing in both directions.

Construction of Env mutants

To study the effects of specific amino acid mutations, at residues within or near the 2F5 epitope on neutralization sensitivity, we introduced site-directed mutations into the Env clones 14/00/4, 24/00/4, NYU1026 and NYU1423 using the Quick Change mutagenesis kit (Stratagene, La Jolla, CA). Mutations were verified by sequencing.

Acknowledgments

This study was supported by NIH Grant AI37438 awarded to Dr. G Quinnan and Grant number G.0302.01 of the Fund for Scientific Research Flanders (Belgium). The authors are grateful to Drs. S. Zolla-Pazner and P. Nyambi of the Immunology Core of the NYU center for AIDS Research (AI 27742) for providing the NYU samples. We acknowledge M. Flora for assistance with sequencing of the envelopes and Ms. K. Vereecken, L. Gong and S. Dey for excellent technical assistance.

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