Available online at www.sciencedirect.com

SCIENCE DIRECT

Virology 337 (2005) 136 – 148

VIROLOGY

www.elsevier.com/locate/yviro

Neutralization sensitivity of HIV-1 Env-pseudotyped virus clones is determined by co-operativity between mutations which modulate the CD4-binding site and those that affect gp120–gp41 stability

Simon Beddows[a,b,*], Natalie N. Zheng[c], Carolina Herrera[b], Elizabeth Michael[b], Kelly Barnes[b], John P. Moore[b], Rod S. Daniels[c], Jonathan N. Weber[a]

[a]*Jefferiss Research Trust Laboratories, Wright Fleming Institute, Imperial College School of Medicine, London W2 1PG, UK*
[b]*Department of Microbiology and Immunology, Weill Medical College, Cornell University, New York, NY 10021, USA*
[c]*Virology Division, National Institute for Medical Research, The Ridgeway, London NW7 1AA, UK*

Received 4 January 2005; returned to author for revision 22 February 2005; accepted 28 March 2005
Available online 26 April 2005

Abstract

Adaptation of antibody neutralization-resistant human immunodeficiency virus type I (HIV-1) to growth in vitro generally results in the acquisition of a neutralization-sensitive phenotype, an alteration of viral growth kinetics, and an array of amino acid substitutions associated with these changes. Here we examine a panel of Env chimeras and mutants derived from these neutralization-resistant and -sensitive parental Envs. A range of neutralization and infectivity phenotypes was observed. These included a modulation of the CD4 binding site (CD4bs) towards recognition by neutralizing and non-neutralizing CD4bs-directed antibodies, resulting in a globally neutralization-sensitive Env; alterations which affected Env complex stability; and interactions which resulted in differential infectivity and CCR5/CXCR4 usage. This range of properties resulted from the complex interactions of no more than three amino acids found in key Env locations. These data add to a growing body of evidence that dramatic functional alterations of the native oligomeric Env protein complex can result from relatively minor amino acid substitutions.

Keywords: HIV; Envelope; gp120; gp41; Antibody; Neutralization; Mutants; CD4 binding site

Introduction

The generation of a broadly neutralizing antibody response remains a major goal in the development of a human immunodeficiency virus type 1 (HIV-1) vaccine, in addition to other immune effector mechanisms (Garber et al., 2004; Klausner et al., 2003). However, natural envelope polymorphisms, poor presentation, and/or immunogenicity of defined neutralizing antibody epitopes, the potential for 'escape' from neutralizing antibodies, and neutralization resistance in general have all greatly hampered vaccine strategies to date (Burton et al., 2004). Neutralizing monoclonal antibodies (MAbs) have been characterized as interfering with attachment to CD4; disrupting gp120/CD4/co-receptor interactions; or preventing fusion with the target cell membrane (Burton et al., 1994, 2004; Conley et al., 1994; Muster et al., 1993; Thali et al., 1991a, 1993; Trkola et al., 1996; Zwick et al., 2001). While antibodies with these specificities are reactive against a diverse range of isolates (Binley et al., 2004; D'Souza et al., 1997; Trkola et al., 1995), they appear to be rarely generated during natural infection. Broadly cross-neutralizing antibodies can be generated during natural infection, albeit to low titer (Moore et al., 1996; Nyambi et al., 1996; Weber et al., 1996), but their specificity has rarely, if ever, been fully defined. Encouragingly, passive transfer studies using MAbs or polyclonal antisera provide a proof of principle that pre-existing neutralizing antibodies can significantly protect against HIV/SHIV chal-

* Corresponding author. Department of Microbiology and Immunology, Weill Medical College, Cornell University, New York, NY 10021, USA. Fax: +1 212 746 8340.
E-mail address: sib2005@med.cornell.edu (S. Beddows).

0042-6822/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2005.03.033

lenge (Conley et al., 1996; Mascola et al., 1999, 2000; Shibata et al., 1999; Veazey et al., 2003).

In one approach to defining novel neutralizing antibody epitopes for vaccine exploitation, several groups are trying to define viral envelope alterations that confer differential neutralization sensitivity or resistance. Such alterations arise from passage of laboratory-adapted or clinical HIV-1 isolates in vitro or in vivo, in some cases under antibody selection pressure. They have been reported to affect neutralization sensitivity of HIV-1 strains to individual MAbs or more globally to a range of antibodies directed to diverse epitopes (Cayabyab et al., 1999; Cheng-Mayer et al., 1999; Follis et al., 1998; Mo et al., 1997; Park et al., 1998; Pugach et al., 2004; Reitz et al., 1988; Sawyer et al., 1994; Watkins et al., 1996; Wrin et al., 1995). While some of these alterations were found in defined neutralizing antibody epitopes and affect antibody binding directly, many were found at distant residues and are presumed to confer neutralization sensitivity or resistance indirectly, possibly by changing the overall structure of the HIV-1 envelope.

In a previous study, we described the differential neutralization sensitivity of a peripheral blood mononuclear cell (PBMC)-derived R5X4 HIV-1 strain (W61D) and its T-cell line adapted (TCLA) counterpart to HIV-1-specific MAbs, HIV-1 patient sera, and envelope-homologous vaccinee sera, despite the absence of any changes in V1, V2, and V3 (Beddows et al., 1999). We have now generated full-length *env* clones from the PBMC and TCLA virus stocks, in addition to the construction of 5 chimeras and 10 mutants, to elucidate the mechanisms of this differential neutralization sensitivity. We demonstrate that the altered neutralization phenotypes for HIV-1_{W61D} during T-cell line adaptation are due to conformational changes within the native Env oligomer rather than alteration of specific antibody epitopes. Structure–function studies such as these, especially where co-operativity between Env domains are implicated, should help to elucidate how neutralization resistance occurs, and contribute to our understanding of critical targets for vaccine development.

Results

Parental Env variants display a range of neutralization sensitivities to HIVIg

We generated parental *env*-gene clones from acutely infected PBMC (HIV-$1_{W61D/PBMC}$) and SupT1 (HIV-$1_{W61D/SupT1}$) cells using the same virus stocks that have been previously described to display differential neutralization sensitivities to a range of anti-Env antibodies (Beddows et al., 1999). In all, seven full-length functional *env*-genes were rescued; four from the HIV-$1_{W61D/PBMC}$-infected cells (7.1, 7.4, 7.12, and 7.15) and three from HIV-$1_{W61D/SupT1}$-infected cells (6.7, 6.14, and 6.24). Env-pseudotyped virus bearing the encoded Envs were able to infect both CXCR4- and CCR5-expressing U87.CD4 cells, recapitulating the parental R5X4 biological phenotype (data not shown). In order to assess whether the parental Env variants also displayed the differential neutralization sensitivity apparent in the virus stocks from which they were derived, we first tested Env-pseudotyped virus stocks against the Ig-purified polyclonal reagent, HIVIg (Fig. 1). Overall, the SupT1-derived clones were more neutralization sensitive ($P < 0.01$; Mann–Whitney U test) than the PBMC-derived clones, though the distribution of neutralization titers between the two groups did overlap. For example, two of the three SupT1-derived clones (6.7 [mean ± SD IC_{50}, 6 ± 4 μg/ml; $n = 4$] and 6.14 [71 ± 8 μg/ml]) and one PBMC-derived clone (7.15 [137 ± 98 μg/ml]) were relatively neutralization sensitive, while one PBMC clone (7.12 [923 ± 117 μg/ml]) was particularly neutralization resistant. Indeed, Env-pseudotyped virus expressing the envelope from clone 6.7 was 154-fold more sensitive to neutralization than the pseudovirus derived from clone 7.12. The remaining three clones (6.24, 7.1, and 7.4) were of intermediate sensitivity, displaying a 2- to 3-fold greater sensitivity to HIVIg than clone 7.12.

env-gene sequencing analysis

Each parental *env*-gene clone was fully sequenced and Env translation products were aligned with the amino acid sequence of the neutralization resistant clone, 7.12, to allow a better assessment of what polymorphisms may have accounted for the observed differential neutralization sensitivity (Fig. 1). One substitution, E440G (numbered according to the HXB2 Env sequence), was present in 3/3 SupT1 clones but 0/4 PBMC clones, suggesting it had arisen during T cell line adaptation. Three changes, D241N, N636D, and K805Q, were found in all clones except 7.12, suggesting that one or more of them may modulate neutralization sensitivity in the remaining Envs. However, the 1–2 $\log_{10}$ increase in neutralization sensitivity to HIVIg displayed by clones 6.7, 6.14, and 7.15 was unlikely to be due to these polymorphisms alone. Three of the Env substitutions (N188K, D241N, and N616K) would be expected to alter potential N-linked (NXS/T) glycosylation sites. The neutralization-sensitive clone 6.7 had a substitution (D457G) in a position involved in gp120 binding to CD4 (Kwong et al., 1998), a substitution in the V3 domain (A316T), and a substitution in the leucine zipper-(LZ)-like domain in gp41 (H564N) (Douglas et al., 1997). This latter substitution was also found in clone 6.24, which displayed intermediate neutralization sensitivity to HIVIg. Indeed, all three SupT1-derived clones and the neutralization sensitive PBMC-derived clone, 7.15, had one or more substitutions in, or adjacent to, this gp41 domain.

Env glycoprotein determinants of neutralization sensitivity

In an attempt to delineate the impact that one or more of these amino acid substitutions may have on the overall neutralization sensitivity of the W61D Env, we initially

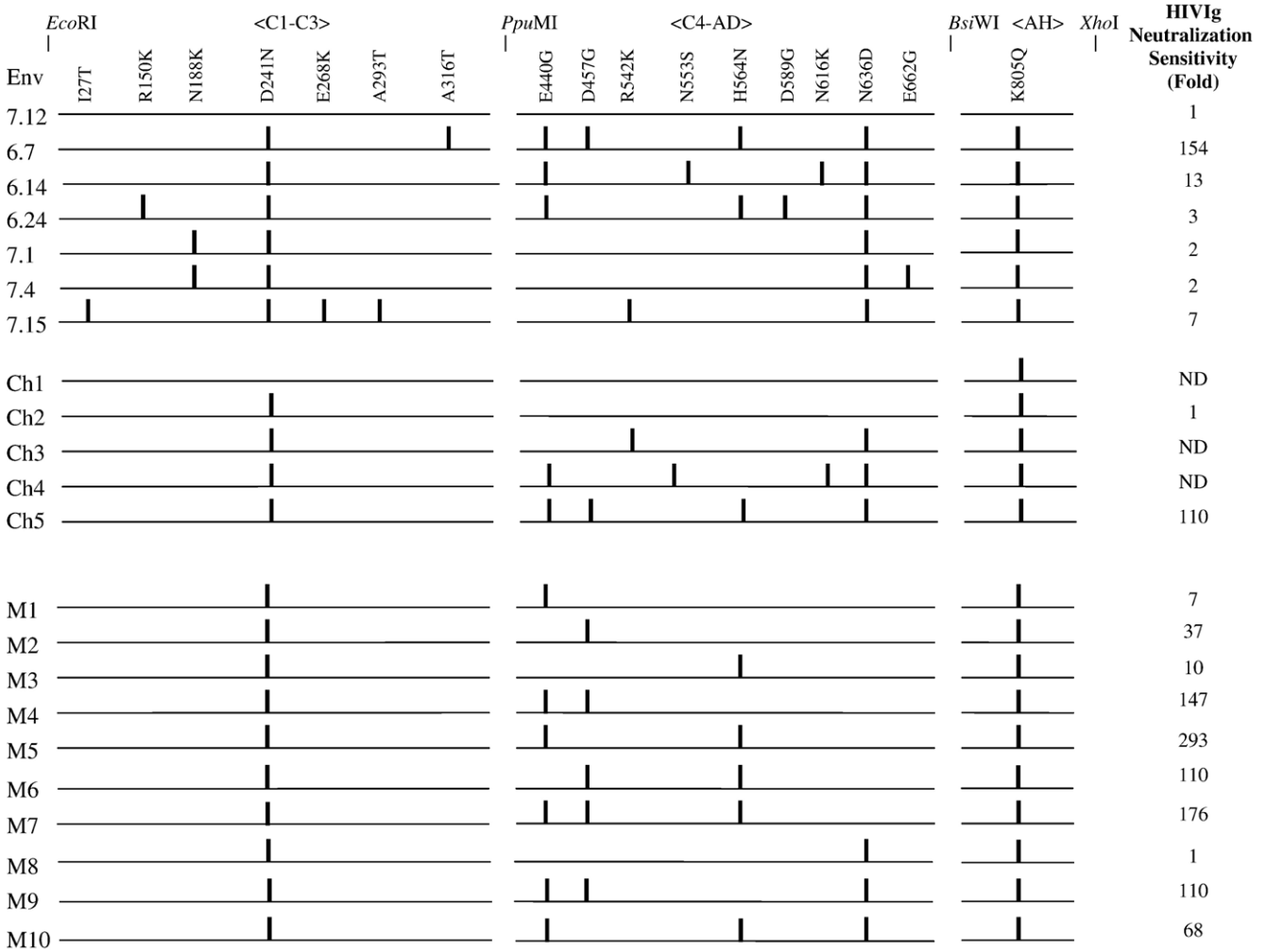

Fig. 1. Amino acid sequences of the full-length parental Env clones derived from the PBMC (7.1, 7.4, 7.12, and 7.15) or TCLA (6.7, 6.14, and 6.24) stocks of W61D and the recombinant chimeric (Ch) and mutant (M) Envs derived thereof. The positions of the restriction enzymes *Eco*RI, *Ppu*MI, *Bsi*WI, and *Xho*I are noted, as are the resulting Env domains contained in the fragments following enzyme digestion. C refers to the indicated gp120 constant domain, AD refers to the assembly domain of gp41, and AH refers to the amphipathic α-helices (Douglas et al., 1997). Env polymorphisms were numbered according to the residues in the HIV-1 HXBc2 Env, in line with current convention. All sequences are compared to the neutralization resistant Env clone 7.12, with differences denoted by a vertical bar at the indicated position. Neutralization values for each Env-pseudotyped virus are presented as the fold increase in neutralization sensitivity as a function of the mean HIVIg IC_{50} derived from 2–4 experiments. Note that since the neutralization data generated for the parental Env clones were determined using a different stock of HIVIg than that for the chimeras or mutants, the derived values presented are relative to parental Env 7.12 (6.7, 6.14, 6.24, 7.1, 7.4, and 7.15) or chimeric Env Ch2 (Ch5, M1–M10). ND, not done.

generated chimeric Envs making use of the naturally occurring restriction enzyme sites, *Ppu*MI and *Bsi*WI (Fig. 1). We reasoned that the amino acid substitutions present between the C4 domain of gp120 and the assembly domain of gp41 found in clones 6.7, 6.14, and 7.15 would most likely recapitulate these neutralization phenotypes. Indeed, the chimeric Env-pseudotyped viruses Ch1/Ch2 (7.12), Ch3 (7.15), Ch4 (6.14), and Ch5 (6.7) displayed a neutralization phenotype almost identical to that of the indicated parental clone on which they were based, when tested in neutralization assays using a polyclonal HIV-1-positive human serum (data not shown). For example, both the Ch5 chimera and clone 6.7 (90% neutralizing titer of >320) were substantially more neutralization sensitive than chimera Ch2 and clone 7.12 (90% neutralizing titer, 10). These data confirmed that the neutralization phenotype displayed by these two chimeras, at least against this polyclonal human HIV-1-positive serum, was conferred by the interaction of no more than the four amino acid substitutions; E440G, D457G, H564N, and N636D. The D241N and K805Q substitutions, therefore, did not appear to impact on the neutralization sensitivity or resistance of these Env clones.

A more detailed assessment of the neutralization sensitivity or resistance of these two chimeric Env-pseudotyped viruses, Ch2 and Ch5, suggested these four amino acid substitutions confer a globally neutralization sensitive phenotype against a panel of monoclonal and polyclonal antibodies (Fig. 2). However, while Ch5 was clearly more neutralization sensitive than Ch2 to a polyclonal serum preparation (HIVIg; see also Fig. 1), to a V3-specific MAb (447-52D), to two CD4bs-directed MAbs (F105 and 205-46-9), and, marginally, to a MAb with a neutralization

determinant in gp41 (2F5), this was not the case for the gp120-specific MAb 2G12 (which recognizes a unique mannose-dependent epitope), nor for two other CD4bs-directed reagents (CD4-IgG2 and b12).

To further define the interaction of these four amino acid substitutions, we constructed a panel of 10 mutant Envs (designated M1–M10). In these mutants, we used site-specific mutagenesis to introduce each substitution individually, or in combination, into the Ch2 background (Fig. 1). Two of the parental clones (6.7 and 7.12), the chimeras Ch2 and Ch5, and the mutant Envs (M1–M10) were then tested against a single concentration of anti-Env antibody preparation in neutralization assays (Fig. 3). While this approach is useful for screening large numbers of (pseudo)viruses and antibodies (Si et al., 2001), we do recognize the limitations of testing for neutralization efficacy using a single concen-

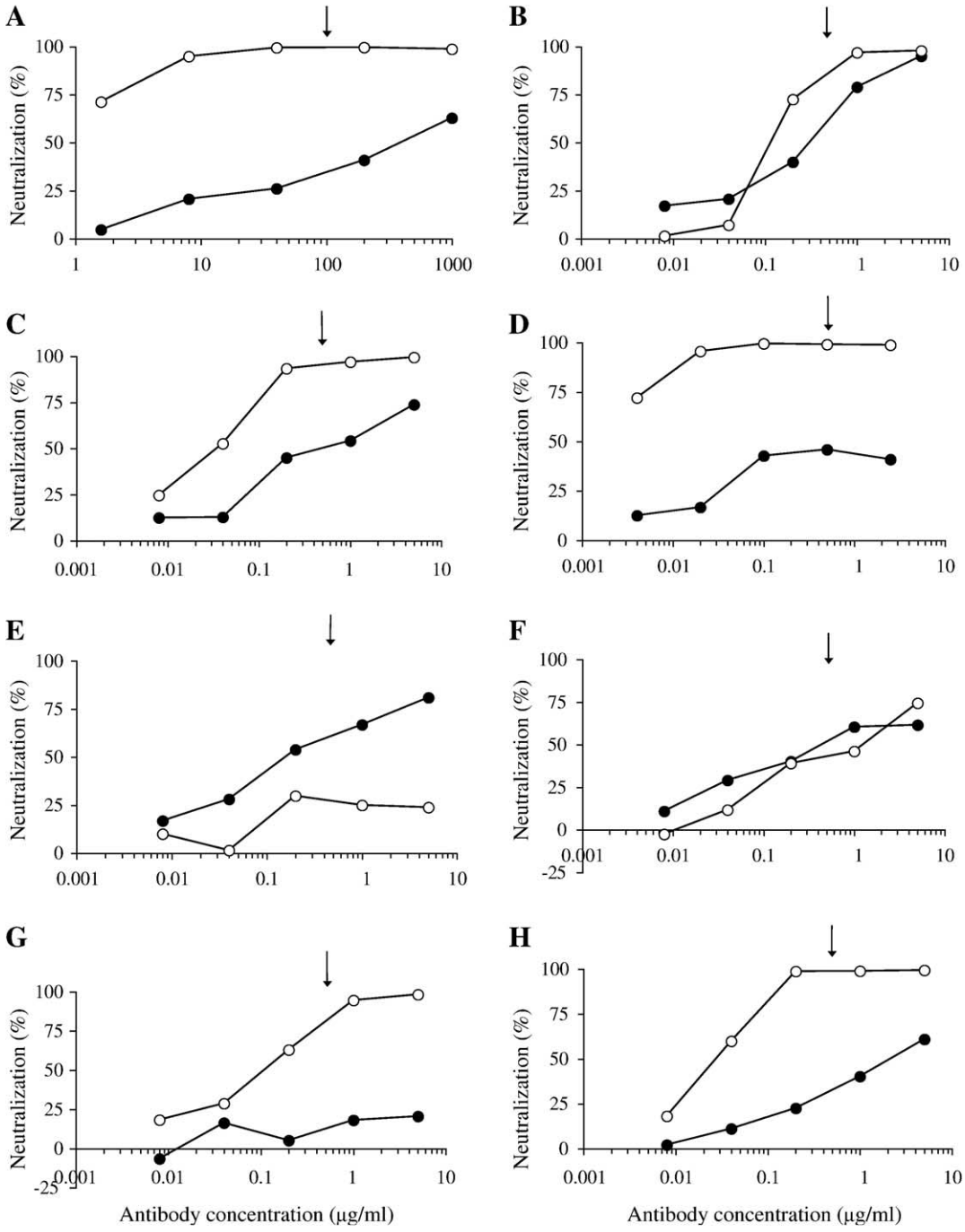

Fig. 2. Neutralization sensitivity of Ch2 (filled circles) and Ch5 (open circles) chimeric, Env-pseudotyped HIV-1 stocks by a panel of Env-specific neutralization reagents: A, HIVIg; B, 2G12; C, 2F5; D, 447-52D; E, CD4-IgG2; F, b12; G, F105; H, 205-46-9. Inhibition of infection was performed on U87.CD4.CXCR4 cells in the presence of increasing amounts of the indicated antibody (μg/ml, *x*-axis). The arrow indicates the antibody concentration used in Fig. 3. Data are representative of two experiments.

tration of antibody. We do not consider individual comparisons where there is <2-fold difference to be significant by this method.

The neutralization sensitivity profiles of the chimeric Envs, Ch2 and Ch5, to these monoclonal and polyclonal Env-specific reagents (Fig. 2), were effectively represented using the single concentration of neutralizing reagent in this assay format (Fig. 3). The mutant viruses containing the individual changes E440G (M1), H564N (M3), and N636D (M8) were marginally more neutralization sensitive to HIVIg than Ch2 (Fig. 3). The E440G/H564N (M5) double mutant and the E440G/H564N/N636D (M10) triple mutant displayed greater neutralization sensitivity to HIVIg, relative to Ch2. Indeed, the marginal increases in sensitivity seen with

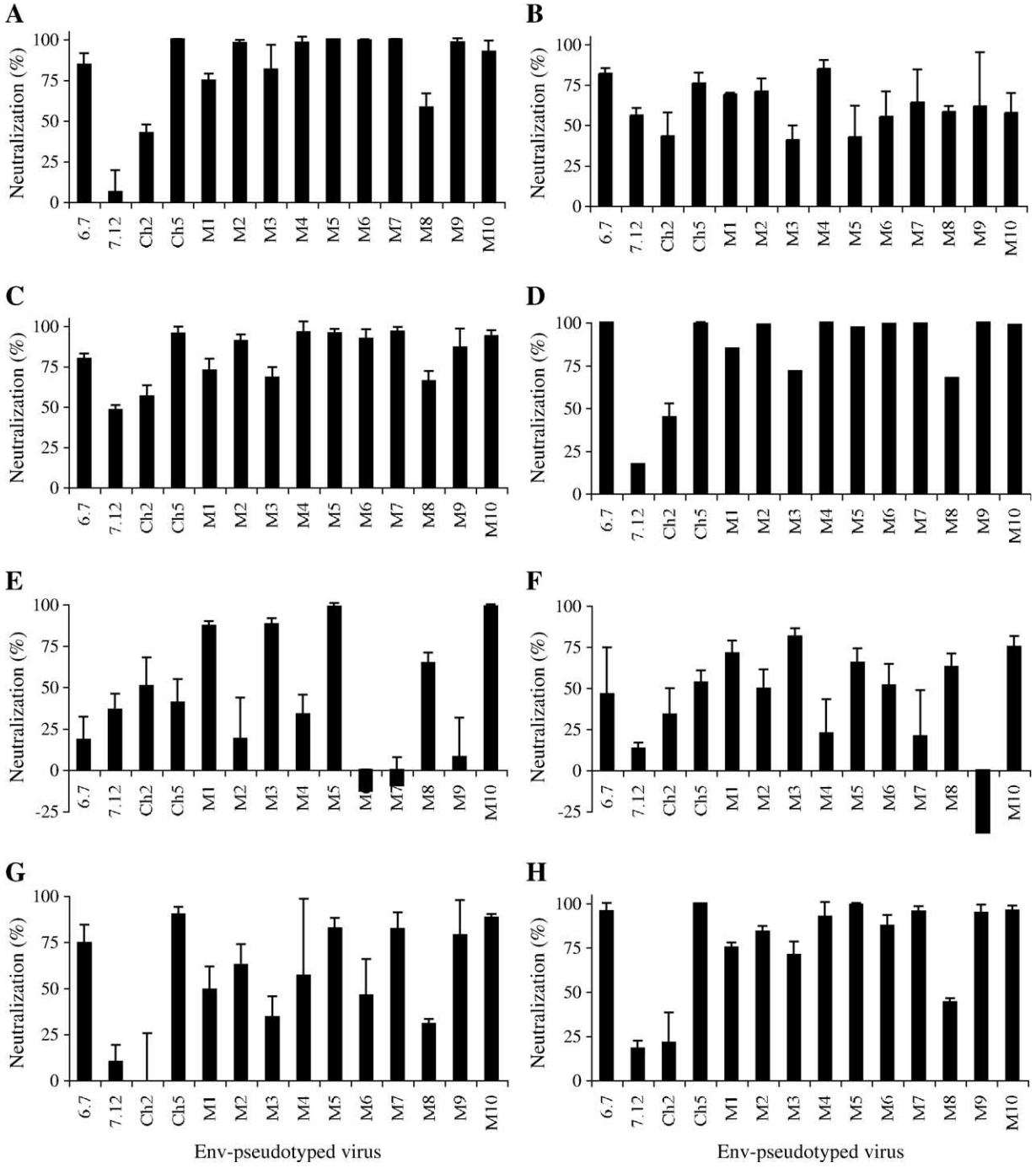

Fig. 3. Neutralization sensitivity of parental (6.7 and 7.12), chimeric (Ch2 and Ch5), and mutant (M1–M10) Env-pseudotyped HIV-1 stocks against a panel of Env-specific antibodies: A, HIVIg; B, 2G12; C, 2F5; D, 447-52D; E, CD4-IgG2; F, b12; G, F105; H, 205-46-9. The concentration used for polyclonal HIVIg was 100 μg/ml, and 0.5 μg/ml for the monospecific reagents. Bars represent the mean (±SD) percentage neutralization derived from three experiments, except for the parental and mutant Envs against 447-52D, which represent the mean of two experiments.

mutants M1, M3, and M8 in this assay format, and the greater neutralization sensitivity displayed by M5 and M10 Envs, are consistent for most of the antibodies used.

Env-pseudotyped viruses containing the D457G mutation (6.7, Ch5, M2, M4, M6, M7, and M9) were also more sensitive to HIVIg, 2F5, and 447-52D neutralization than Ch2. Envs containing this mutation were markedly resistant to b12 and CD4-IgG2, while their sensitivity to F105 and 205-46-9 was unaffected or even enhanced, relative to mutants lacking this substitution. Comparisons of M5 with M7 and Ch5 and M10 highlight the differential effects of the D457G mutation on recognition of the CD4bs by these CD4bs-directed reagents.

To extend these observations, we tested the sensitivity of the chimeric Envs Ch2 and Ch5, and mutant Env-pseudotyped viruses (M1–M10), to HIVIg and two CD4bs-directed reagents, 205-46-9 and CD4-IgG2, in a more traditional assay in which the antibodies were titrated against input pseudovirus (Table 1 and Fig. 1). This confirms the minor increase in neutralization sensitivity to all three reagents conferred by the individual mutations E440G (M1; a 6.7-fold increase in sensitivity to HIVIg over that obtained for Ch2) and H564N (M3; 9.7-fold), the at least additive effect displayed by the double mutant E440G/H564N (M5; 291-fold), and the lack of an apparent effect of the N636D (M8; 1.1-fold) mutation. These data also confirm the differential effect of the D457G mutation on sensitivity to antibodies directed against the CD4 binding site on gp120. There were, however, some minor discrepancies between the two assay systems. For example, M8 Env-pseudotyped virus appeared to be more sensitive than Ch2, and marginally less sensitive than M1, to neutralization by HIVIg in the single concentration assay (Fig. 3). In the more robust assay system using a serial dilution of antibody, the M8 Env-pseudotyped virus was essentially as resistant as Ch2, and M1 was 6-fold more sensitive to this reagent (Table 1 and Fig. 1). Thus, the single concentration neutralization assay was used to generalize on patterns of reactivity only, and more weight should be applied to neutralization data derived using the antibody dilution assay.

Antigenicity assessment of W61D Envs

To determine whether the differential neutralization sensitivity of the Env clones was due to sequence polymorphisms within the epitopes of the antibodies, or an indirect effect on exposure of these epitopes on the native Env oligomer, we assessed the ability of these antibodies to bind monomeric gp120 derived from lysed Env-pseudotyped virion preparations. The binding of polyclonal HIVIg or the CD4bs-directed MAbs, 205-46-9 and F105, to these Env-pseudotyped-derived gp120s in an ELISA was unaffected by any of these amino acid substitutions (Figs. 4A and B, and data not shown). In contrast, the binding of the CD4-IgG2 reagent and MAb b12 towards any gp120 that contained the D457G mutation (6.7, Ch5, M2, M4, M6, M7, and M9) was severely disrupted (Fig. 4C, and data not shown). A similar result was obtained using soluble monomeric gp120 proteins expressed in vitro, rather than derived from lysed pseudovirions (data not shown). The limitations of a gp120-based assay system notwithstanding, these results suggest that of the possible gp120 polymorphisms found within these Envs, only the D457G mutation directly modifies the binding site for any of the test reagents. Hence neutralization sensitivity to these reagents must be conferred indirectly, for example, by alteration of the exposure of gp120 epitopes by a combination of gp120 and gp41 sequence changes.

Relationship between infectivity and neutralization sensitivity

During the course of these studies, it was apparent that when the stocks were titrated on susceptible co-receptor

Table 1
Neutralization of pseudotyped HIV-1_{W61D} bearing chimeric and mutant Envs[a]

Env	Polymorphisms	HIVIg		CD4IgG2		205-46-9	
		50%	90%	50%	90%	50%	90%
Ch2		87.5 (25)	>100	0.22 (0.11)	>1	>1	>1
Ch5	E440G/D457G/H564N/N636D	0.8 (0.8)	3.9 (2.8)	0.76 (0.48)	>1	0.02 (0.01)	0.11 (0.01)
M1	E440G	13	>100	0.05	1	0.1	>1
M2	D457G	2.4	18	>1	>1	0.07	>1
M3	H564N	9	>100	0.3	0.9	0.11	>1
M4	E440G/D457G	0.6	20	>1	>1	0.008	0.2
M5	E440G/H564N	0.3	0.7	0.04	0.5	0.02	0.2
M6	D457G/H564N	0.8	13	>1	>1	0.001	0.06
M7	E440G/D457G/H564N	0.5	8.3	>1	>1	0.03	0.6
M8	N636D	83	>100	0.25	>1	>1	>1
M9	E440G/D457G/N636D	0.8	10	>1	>1	0.08	0.8
M10	E440G/H564N/N636D	1.3	13	0.01	0.3	0.05	0.2

[a] Numbers indicate mean (SD) of 4 determinations for Env-pseudotypes Ch2 and Ch5, or representative data from two experiments for the mutants M1–M10, in μg/ml for each neutralizing reagent at the indicated neutralization endpoint (50% or 90%).

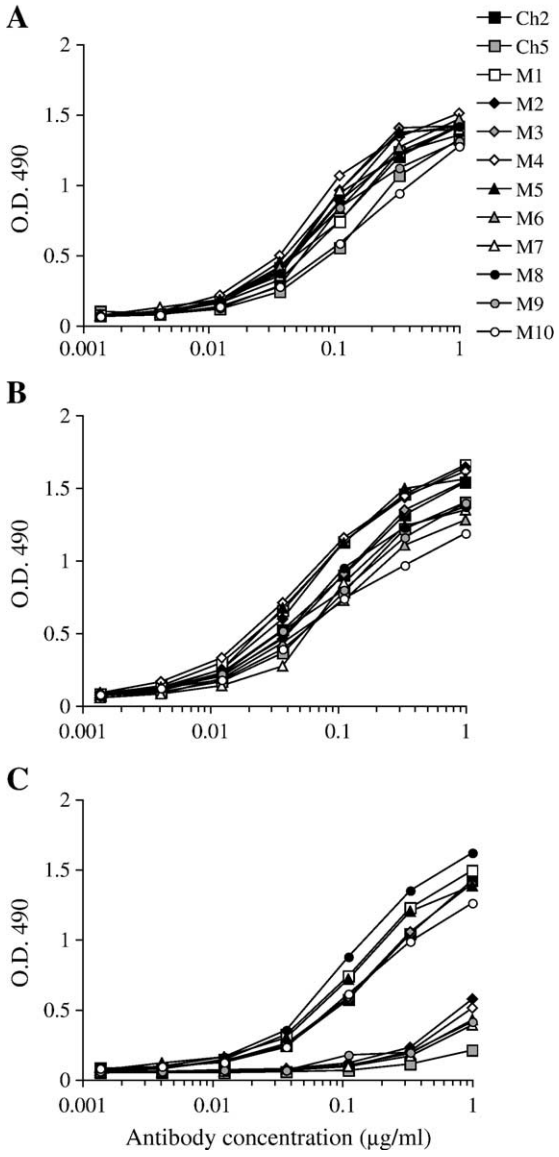

Fig. 4. Antibody binding to Env-pseudotyped virus-derived gp120 by ELISA. The indicated concentrations of (A) HIVIg, (B) 205-46-9, and (C) CD4-IgG2 were allowed to bind to solubilized pseudovirion-derived gp120 before being resolved with anti-human conjugated antibody. Data are representative of that derived from several experiments.

bearing cells, clone 6.7 and chimera Ch5 generated less luciferase activity than clone 7.12 or Ch2, although for neutralization studies the amount of input virus was normalized by luciferase output to reflect this disparity. A relationship between high infectivity and neutralization resistance has previously been demonstrated (Leavitt et al., 2003; Park and Quinnan, 1999). Hence we wished to assess any possible similar relationship with our recombinant Envs. To do so, supernatant from Env-pseudotyped Ch2 and Ch5 chimeras, and all 10 mutants, were titrated on U87.CD4.CXCR4 cells and the luciferase content determined after 3–4 days. Chimeric or mutant Env-pseudotyped viruses containing the E440G, D457G, or H564N mutations were 20- to 481-fold less infectious than the Ch2 chimeric Env-pseudotyped virus, suggesting that these mutations alone or in combination impaired the ability of virus to bind or enter susceptible target cells (Fig. 5A). Env-pseudotyped viruses containing both the E440G and D457G mutations, M4 and M9, were the least infectious, being 316-fold and 481-fold less infectious than Ch2, respectively. The N636D mutation (M8) displayed similar infectivity to that observed with Ch2. There was a significant correlation between the infectivity (TCID50) of the Env-pseudotyped virus stocks generated by transient transfection of 293T cells and the resultant neutralization sensitivity of these Env-pseudotyped virus stocks to the polyclonal reagent, HIVIg (Spearmans Rank Correlation, $P < 0.01$). However, as the Env-pseudotyped virus stocks were normalized to approximately the same level of infectivity for the neutralization assay, and neutralization by a polyclonal reagent such as HIVIg is likely to be mediated by antibodies with varying affinities for each Env, such a simplistic association should be treated with some caution.

It was possible that the differences in infectivity noted here were simply due to a reduced output of Env-pseudotyped virus; thus, we attempted to relate Env-pseudotype infectivity as a function of pseudovirion particle output (p24 content). To do so, we first had to separate virions from free p24 that would confound such measurements. However, when the various supernatants were concentrated through a sucrose cushion and tested against CXCR4-bearing cells, only some of the chimeras and mutants generated the expected (given the volume adjustments) 10-fold increase in luciferase output. Indeed, chimera Ch2 and all other mutants that lacked the H564N mutation (M1, M2, M4, M8, and M9) were less infectious after purification; their luciferase output was reduced by 1–2 $\log_{10}$ (18- to 447-fold reduction; Fig. 5B). This was an unexpected finding, since sucrose cushion centrifugation does not affect HXB2 or JR-FL Env-pseudotyped HIV-1 in this way (data not shown). The reduction in infectivity was not due to a reduced recovery of virus particles, because p24 antigen and RT content of the various sucrose-pelleted samples were similar (data not shown). Also, the Env and Gag contents of sucrose cushion-concentrated Ch2 and Ch5 Env-pseudotyped HIV-1 were seemingly unaffected by sucrose cushioning, as assessed by Western blotting (data not shown). In addition, determination of Env-pseudotyped virus spike density following S1000 column separation of virions from soluble proteins (O'Brien et al., 1994) suggested that the Ch2 and Ch5 chimeric Env-pseudotype viruses contained similar amounts of gp120 (data not shown).

The simplest explanation of these seemingly contradictory observations is that the Ch2 chimeric fusion-competent Env spikes are somehow functionally impaired by ultracentrifugation (but not by S-1000 chromatography, a gentler technique). One way to test this possibility was to assess whether Ch2 sheds gp120 more readily than the Ch5 chimera. When we assessed the gp120 content of the test viruses, we found that sucrose-pelleted Ch2 chimeric Env-

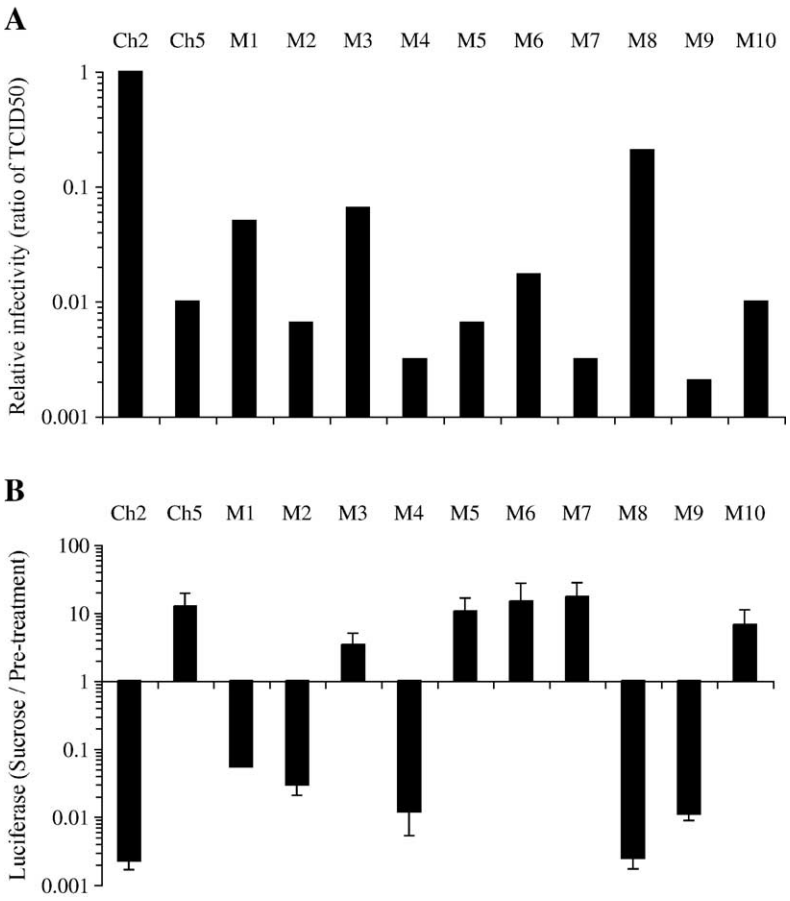

Fig. 5. Relative impact of Env polymorphisms on Env-pseudotyped virus infectivity. Culture supernatant containing Env-pseudotyped HIV-1 was generated by transient transfection of HEK 293T cells and relative infectivity was determined. (A) TCID50 measurements of untreated supernatant were normalized against those obtained for chimera Ch2 and represent the mean of two such determinations. (B) Relative infectivity, using luciferase RLU, was determined for each Env-pseudotyped virus prior to and following concentration through a 20% sucrose cushion and represent the mean (±SD) of data derived from 2–3 experiments.

pseudotyped virus treated at room temperature with or without sCD4 shed ~3–4 times more gp120 than the similarly treated Ch5 chimera (data not shown). Thus, at least one potential explanation of this phenomenon appeared to be gp120 instability on the Env-pseudotyped virus.

All these clones, chimeras, and mutants could utilize either CXCR4 or CCR5 for entry, although there were minor differences in the efficiency of co-receptor usage for mutants that did not contain the H564N mutation. The latter mutant Env-pseudotyped viruses replicated on U87.CD4.CXCR4 cells approximately twice as efficiently (mean 1.92, SD 0.48) as on U87.CD4.CCR5 cells, while Ch5 and mutants containing the H564N mutation replicated on U87.CD4.CXCR4 cells less efficiently than on U87.CD4.CCR5 cells (mean 0.88, SD 0.33) (data not shown).

The above studies suggest that any relationship between infectivity and neutralization sensitivity of these Env clones is likely to be complex and influenced by multiple factors. The infectivity profiles of mutants containing or lacking the H564N mutation suggest a further role for this change in modulating pseudovirion infectivity, perhaps by stabilization of the pseudovirion-associated Env complex, as judged by the gp120 shedding experiments.

Discussion

While cellular adaptation and a concomitant increase in neutralization sensitivity are not novel phenomena, the mechanisms by which HIV-1 can adapt to its surroundings often involve dramatic structural re-arrangements within the Env complex. The interplay of these changes on the associated viral phenotype is worthy of investigation. In this study, we attempted to determine the impact of Env amino acid polymorphisms on the differential neutralization sensitivity of a PBMC-derived HIV-1 strain ($HIV\text{-}1_{W61D/PBMC}$) and its T-cell line adapted counterpart ($HIV\text{-}1_{W61D/SupT1}$) (Beddows et al., 1999). To this end, we generated seven functional full-length Env clones, five chimeric Envs, and a panel of ten mutant Envs, then studied their neutralization sensitivity to polyclonal and monoclonal Env-specific reagents, Env stability, and their infectivity as Env-pseudotyped viruses. Diverse infectivity

and neutralization phenotypes were conferred by the interactions of no more than three amino acid substitutions (E440G, D457G, and H564N).

In addition to these major substitutions, additional sequence changes may have played a minor role in influencing the neutralization sensitivity of some individual clones with intermediate neutralization sensitivity. One substitution, N188K, present in clones 7.1 and 7.4, disrupts the same potential N-linked glycosylation site as one of those reported to arise in a CD4-independent Env clone derived from HXBc2 (LaBranche et al., 1999). The D589G substitution (clone 6.24) was at one of the positions reported to affect membrane fusion and virus infectivity (Cao et al., 1993), and the N616K (clone 6.14) substitution would be predicted to disrupt another potential N-linked glycosylation site. The effect of these substitutions is unclear, and we did not directly test them. Of the three other mutations, one, D241N, also affected a potential N-linked glycosylation site. The N636D and K805Q mutations were present in all Envs except the most neutralization-resistant ones. A logical assumption was that these two changes might have a significant influence on neutralization sensitivity. However, we could find no experimental evidence to support this supposition; the changes may be inconsequential, or they might have a minor, undetectable effect.

One substitution, E440G, appeared to be associated with adaptation on cell lines, in that it was present in all SupT1 clones but none of the PBMC clones. Substitutions at or near this position have been reported to reduce HIV-1_{YU-2} gp120 binding to CCR5, reduce Env-pseudotype infectivity via CCR5, and modulate sensitivity to entry inhibitors, although neutralization sensitivity to antibodies was not tested (Reeves et al., 2004; Rizzuto et al., 1998). In the present study, the E440G mutation reduced infectivity to both CXCR4- and CCR5-mediated entry and bestowed a modest increase in sensitivity to Env-specific antibodies.

Another substitution, D457G, which had a significant impact on virus infectivity and neutralization sensitivity was located in a position involved in binding of gp120 to CD4 (Kwong et al., 1998). Consistent with its location, this change impaired the gp120–CD4 interaction at the level of monomeric gp120. Substitutions at this position have also been reported to affect the binding of the neutralizing antibody b12, but not the non-neutralizing b6 antibody, to gp120 (Pantophlet et al., 2003; Roben et al., 1994). In keeping with these observations, the neutralization sensitivity and gp120 binding profiles of the D457G-containing mutants toward CD4bs-directed reagents differed depending on whether those reagents are considered to be able to neutralize primary HIV-1 isolates (CD4-IgG2 and b12) or not (F105 and 205-46-9) (D'Souza et al., 1997; Moore et al., 1995). Thus, our data confirm that this single amino acid substitution can modulate the interaction of the CD4 binding site with such neutralizing and non-neutralizing antibodies to different extents.

The third mutation that we focussed on, H564N, is located in the LZ-like domain of gp41. It was present in two SupT1-derived clones (6.7 and 6.24), while a third TCLA clone (6.14) and one of the neutralization-sensitive PBMC-derived Envs (7.15) also had changes in the same general area of gp41. Mutations in this region have been reported to significantly affect membrane fusion and viral infectivity, especially substitutions disrupting the leucine/isoleucine/valine heptad repeat motif characteristic of the LZ-like domain (Cao et al., 1993; Chen et al., 1993; Dubay et al., 1992; Sanders et al., 2002; Wild et al., 1994). While these residues are not thought to greatly affect Env synthesis, production, or oligomerization, mutations at adjacent residues have been reported to affect gp41–gp120 association and Env processing (Cao et al., 1993). In addition, this mutation is at the same position as one reported to arise in a HIV-1_{MN} clone that escaped a neutralizing HIV-1-positive serum, though notably in the reverse orientation (Asn to His) (Park and Quinnan, 1999; Park et al., 1998, 2000). That the Asn to His mutation arose as a result of neutralizing antibody selection pressure against a TCLA strain (MN), whereas the converse His to Asn change emerged during T-cell line adaptation of a resistant primary virus-like Env is intriguing. Envelope alterations following neutralizing antibody selection and cellular adaptation may therefore be intrinsically linked. While Park et al. noted that this mutation did not appear to have a great impact by itself, it was reported to greatly modulate the effects of other substitutions (Park and Quinnan, 1999; Park et al., 1998, 2000). Similarly, cooperativity between changes in the CD4bs and gp41, and the impact that these have on the sensitivity of HIV-1_{LAI} to the small molecule entry inhibitor, BMS-378806, have recently been demonstrated (Lin et al., 2003).

These findings confirm and extend observations that relatively few amino acid substitutions in key locations can have a profound effect on Env function, including Env-pseudotype infectivity and sensitivity to antibody-mediated neutralization (Cayabyab et al., 1999; Follis et al., 1998; Mo et al., 1997; Park and Quinnan, 1999; Park et al., 1998, 2000; Reitz et al., 1988; Sawyer et al., 1994; Watkins et al., 1996; Wrin et al., 1995). Indeed, the combination of the CCR5 binding site mutation (E440G) and the gp41 mutation (H564N) led to a 1–2 $\log_{10}$ increase in neutralization sensitivity and a decrease in relative infectivity, but without a detrimental impact on Env complex stability. The latter is most likely due to the compensatory effect of the H564N mutation. The inter-relationship between a reduced ability to bind CD4, a decrease in Env-pseudotype infectivity, and an increase in neutralization sensitivity, is exemplified by the partial destruction of the CD4bs by the D457G mutation and the differential binding and neutralization that this mutation confers. One possible explanation of this phenomenon would be that a reduced affinity for CD4 slows the kinetics of viral entry sufficiently to allow neutralizing antibodies more time to bind the functional Env complex and thus tip

the balance towards neutralization and away from infection. A reduction of CCR5 affinity and increased sensitivity to small molecule entry inhibitors has recently been demonstrated for mutants of HIV-1_{YU-2} (Reeves et al., 2004). Also, the apparent instability of the Env complex on the high infectivity, neutralization-resistant Env Ch2-containing pseudovirus might reflect the presence of an altered Env conformation that fuses more rapidly and has a correspondingly reduced susceptibility to neutralizing antibodies. The extra instability of the envelope may be a problem for the virus under certain in vivo conditions, or when the virus is subjected to centrifugation in vitro, but perhaps not under normal cell culture conditions.

Structure–function relationships such as those described here should help to elucidate mechanisms of antibody neutralization resistance and could contribute to our understanding of critical targets for HIV-1 vaccine development and/or the design of small molecule inhibitors of infection.

Materials and methods

Construction of env clones, chimeras, and mutants

Full-length (gp160) proviral DNA *env* sequences were amplified from crude nuclear extracts from acutely infected PBMC (HIV-$1_{W61D/PBMC}$) or SupT1 (HIV-$1_{W61D/SupT1}$) cells (Beddows et al., 1999) using nested PCR primers (Connor et al., 1996), and ligated into the mammalian expression plasmid pcDNA3.1zeo(+) (Invitrogen) using *Eco*RI and *Xho*I restriction enzymes. These engineered enzyme sites (*Eco*RI and *Xho*I) and the naturally occurring sites within these clones (*Ppu*MI and *Bsi*WI) were utilized to generate the chimeric *envs* (Ch1 to Ch5; see Fig. 1) using standard cloning methodologies. The Env mutants (M1–M10; see Fig. 1) were made by site-directed mutagenesis (Stratagene). A stop codon was introduced into position R511* to create the gp120-expressing construct. All Env amino acid positions were numbered according to the residues in the HIV-1 HXBc2 Env, in line with current convention.

Cells and antibodies

The culture conditions for the HEK 293T, U87.CD4.CCR5, and U87.CD4.CXCR4 cell lines have been described previously (Gordon et al., 1999). All cell cultures were maintained at 37 °C in an atmosphere containing 5% CO_2. The epitopes for the anti-HIV antibodies used in this study have been described elsewhere. These include the CD4 binding site-directed MAbs F105 (Thali et al., 1991b), 205-46-9 (formally HT7) (Fouts et al., 1997), and b12 (Burton et al., 1994), the CD4-immunoglobulin G2 (CD4-IgG2) molecule (Trkola et al., 1995), the V3-specific MAb 447-52D (Gorny et al., 1992), the complex carbohydrate-dependent gp120-specific MAb 2G12 (Trkola et al., 1996), and the gp41-specific MAb 2F5 (Muster et al., 1994). HIVIg was obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

Env-pseudotyped virus infectivity and neutralization assays

Pseudotyped virus stocks were produced by calcium phosphate (Promega) co-transfection of 293T cells with each individual pcDNA3.1zeo(+)Env(+) plasmid and the pNL4-3.LUC.R^-E^- reporter plasmid (Connor et al., 1996). Supernatants were clarified by centrifugation and stored in aliquots at −80 °C.

For infectivity assays, Env-pseudotyped virus stocks were serially diluted against U87.CD4.CXCR4 or U87.CD4.CCR5 cells seeded in 48-well trays at a density of 1×10^4/well. Following 3- to 4-day incubation at 37 °C, monolayers were washed with phosphate-buffered saline, lysed in cell culture luciferase lysis buffer, and luciferase activity determined using commercially available reagents (Promega). Env-pseudotyped virus infectivity was determined using the Spearman–Karber estimation of TCID50.

For neutralization assays, Env-pseudotyped virus stocks were normalized to give similar infectious titers against U87.CD4.CXCR4 cells and pre-incubated for 1 h at 37 °C with various anti-Env antibodies, after which time this virus antibody mixture was added to monolayers of U87.CD4.CXCR4 cells. Target cell luciferase activity was assessed 3–4 days post-infection, as above. Neutralizing activity for test wells was determined with reference to control wells containing virus and cells only. In some cases, a specific antibody titer was determined by interpolation using a 50% or 90% neutralization endpoint. As all seven clones were able to use CXCR4 and CCR5 for entry into susceptible target cells, neutralization studies were performed on the same cells throughout; in this case U87.CD4.CXCR4 cells. Several previous studies have demonstrated that antibody-mediated neutralization sensitivity is not affected by specific co-receptor utilization for dual tropic Envs (LaCasse et al., 1998; Montefiori et al., 1998; Trkola et al., 1998).

Antibody binding assay to monomeric gp120

The ability of anti-Env antibodies to bind to solubilized monomeric gp120 prepared from Env-pseudotyped virus stocks lysed with 1% NP40 was performed essentially as described (Trkola et al., 1995), with the exception that pseudovirus stocks were first passed through 0.2-μm filters and pelleted through a 20% sucrose cushion at approximately 100,000 × *g* (Sw41Ti rotor; Beckman) for 1.5 h at 4 °C to remove soluble gp120.

Western blotting

In order to determine the level of Env and Gag expression, pseudovirion-containing supernatant was clari-

fied by filtration through a 0.2-μm filter, then subjected to centrifugation through a 20% sucrose cushion, as described above. The pellet was lysed in SDS-sample buffer containing 100 mM dithiothreitol (Invitrogen), boiled for 3 min, then fractionated on an 8–16% gradient Tris–Glycine gel (Invitrogen), and transferred to a PVDF membrane using standard methodologies. The membranes were subsequently blotted with 2F5 (gp41/gp160) or D7320 (p24; Cliniqa Corp.) antibodies.

Nucleotide sequence accession numbers

The full-length *env* sequence of the parental PBMC clone, W61D 7.12, has been deposited with GenBank (accession number AY973156).

Acknowledgments

We thank Dr. C. Bruck, SmithKline Beecham Biologicals, Belgium, for the original W61D virus stocks. We are grateful to Dr. R.A. Koup and Dr. E. Aarons, formerly of University of Texas SouthWestern Medical Center at Dallas, Texas, for advice and assistance during the initial part of this study. We thank R. Braganza (Imperial College) and L. Campbell-Gardener (Weill Medical College) for excellent technical assistance. S.B. and J.W. were supported by The Wellcome Trust, UK. R.S.D. and N.N.Z. were supported by the British Medical Research Council. This work was supported in part by NIH grants AI 36082 and AI 45463, and by the International AIDS Vaccine Initiative (J.P.M.). J.P.M. is a Stavros S. Niarchos Scholar. The Department of Microbiology and Immunology at the Weill Medical College, Cornell University, gratefully acknowledges the support of the William Randolph Hearst Foundation.

References

Beddows, S., Lister, S., Cheingsong, R., Bruck, C., Weber, J., 1999. Comparison of the antibody repertoire generated in healthy volunteers following immunization with a monomeric recombinant gp120 construct derived from a CCR5/CXCR4-using human immunodeficiency virus type 1 isolate with sera from naturally infected individuals. J. Virol. 73 (2), 1740–1745.

Binley, J.M., Wrin, T., Korber, B., Zwick, M.B., Wang, M., Chappey, C., Stiegler, G., Kunert, R., Zolla-Pazner, S., Katinger, H., Petropoulos, C.J., Burton, D.R., 2004. Comprehensive cross-clade neutralization analysis of a panel of anti-human immunodeficiency virus type 1 monoclonal antibodies. J. Virol. 78 (23), 13232–13252.

Burton, D.R., Pyati, J., Koduri, R., Sharp, S.J., Thornton, G.B., Parren, P.W., Sawyer, L.S., Hendry, R.M., Dunlop, N., Nara, P.L., 1994. Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. Science 266 (5187), 1024–1027.

Burton, D.R., Desrosiers, R.C., Doms, R.W., Koff, W.C., Kwong, P.D., Moore, J.P., Nabel, G.J., Sodroski, J., Wilson, I.A., Wyatt, R.T., 2004. HIV vaccine design and the neutralizing antibody problem. Nat. Immunol. 5 (3), 233–236.

Cao, J., Bergeron, L., Helseth, E., Thali, M., Repke, H., Sodroski, J., 1993. Effects of amino acid changes in the extracellular domain of the human immunodeficiency virus type 1 gp41 envelope glycoprotein. J. Virol. 67 (5), 2747–2755.

Cayabyab, M., Karlsson, G.B., Etemad-Moghadam, B.A., Hofmann, W., Steenbeke, T., Halloran, M., Fanton, J.W., Axthelm, M.K., Letvin, N.L., Sodroski, J.G., 1999. Changes in human immunodeficiency virus type 1 envelope glycoproteins responsible for the pathogenicity of a multiply passaged simian-human immunodeficiency virus (SHIV-HXBc2). J. Virol. 73 (2), 976–984.

Chen, S.S., Lee, C.N., Lee, W.R., McIntosh, K., Lee, T.H., 1993. Mutational analysis of the leucine zipper-like motif of the human immunodeficiency virus type 1 envelope transmembrane glycoprotein. J. Virol. 67 (6), 3615–3619.

Cheng-Mayer, C., Brown, A., Harouse, J., Luciw, P.A., Mayer, A.J., 1999. Selection for neutralization resistance of the simian/human immunodeficiency virus SHIVSF33A variant in vivo by virtue of sequence changes in the extracellular envelope glycoprotein that modify N-linked glycosylation. J. Virol. 73 (7), 5294–5300.

Conley, A.J., Gorny, M.K., Kessler, J.A., Boots, L.J., Ossorio-Castro, M., Koenig, S., Lineberger, D.W., Emini, E.A., Williams, C., Zolla-Pazner, S., 1994. Neutralization of primary human immunodeficiency virus type 1 isolates by the broadly reactive anti-V3 monoclonal antibody, 447-52D. J. Virol. 68 (11), 6994–7000.

Conley, A.J., Kessler II, J.A., Boots, L.J., McKenna, P.M., Schleif, W.A., Emini, E.A., Mark III, G.E., Katinger, H., Cobb, E.K., Lunceford, S.M., Rouse, S.R., Murthy, K.K., 1996. The consequence of passive administration of an anti-human immunodeficiency virus type 1 neutralizing monoclonal antibody before challenge of chimpanzees with a primary virus isolate. J. Virol. 70 (10), 6751–6758.

Connor, R.I., Sheridan, K.E., Lai, C., Zhang, L., Ho, D.D., 1996. Characterization of the functional properties of env genes from long-term survivors of human immunodeficiency virus type 1 infection. J. Virol. 70 (8), 5306–5311.

Douglas, N.W., Munro, G.H., Daniels, R.S., 1997. HIV/SIV glycoproteins: structure–function relationships. J. Mol. Biol. 273 (1), 122–149.

D'Souza, M.P., Livnat, D., Bradac, J.A., Bridges, S.H., 1997. Evaluation of monoclonal antibodies to human immunodeficiency virus type 1 primary isolates by neutralization assays: performance criteria for selecting candidate antibodies for clinical trials. AIDS Clinical Trials Group Antibody Selection Working Group. J. Infect. Dis. 175 (5), 1056–1062.

Dubay, J.W., Roberts, S.J., Brody, B., Hunter, E., 1992. Mutations in the leucine zipper of the human immunodeficiency virus type 1 transmembrane glycoprotein affect fusion and infectivity. J. Virol. 66 (8), 4748–4756.

Follis, K.E., Trahey, M., La Casse, R.A., Nunberg, J.H., 1998. Continued utilization of CCR5 coreceptor by a newly derived T-cell line-adapted isolate of human immunodeficiency virus type 1. J. Virol. 72 (9), 7603–7608.

Fouts, T.R., Binley, J.M., Trkola, A., Robinson, J.E., Moore, J.P., 1997. Neutralization of the human immunodeficiency virus type 1 primary isolate JR-FL by human monoclonal antibodies correlates with antibody binding to the oligomeric form of the envelope glycoprotein complex. J. Virol. 71 (4), 2779–2785.

Garber, D.A., Silvestri, G., Feinberg, M.B., 2004. Prospects for an AIDS vaccine: three big questions, no easy answers. Lancet Infect. Dis. 4 (7), 397–413.

Gordon, C.J., Muesing, M.A., Proudfoot, A.E., Power, C.A., Moore, J.P., Trkola, A., 1999. Enhancement of human immunodeficiency virus type 1 infection by the CC-chemokine RANTES is independent of the mechanism of virus-cell fusion. J. Virol. 73 (1), 684–694.

Gorny, M.K., Conley, A.J., Karwowska, S., Buchbinder, A., Xu, J.Y., Emini, E.A., Koenig, S., Zolla-Pazner, S., 1992. Neutralization of diverse human immunodeficiency virus type 1 variants by an anti-V3 human monoclonal antibody. J. Virol. 66 (12), 7538–7542.

Klausner, R.D., Fauci, A.S., Corey, L., Nabel, G.J., Gayle, H., Berkley, S., Haynes, B.F., Baltimore, D., Collins, C., Douglas, R.G., Esparza, J., Francis, D.P., Ganguly, N.K., Gerberding, J.L., Johnston, M.I.,

Kazatchkine, M.D., McMichael, A.J., Makgoba, M.W., Pantaleo, G., Piot, P., Shao, Y., Tramont, E., Varmus, H., Wasserheit, J.N., 2003. Medicine. The need for a global HIV vaccine enterprise. Science 300 (5628), 2036–2039.

Kwong, P.D., Wyatt, R., Robinson, J., Sweet, R.W., Sodroski, J., Hendrickson, W.A., 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature 393 (6686), 648–659.

LaBranche, C.C., Hoffman, T.L., Romano, J., Haggarty, B.S., Edwards, T.G., Matthews, T.J., Doms, R.W., Hoxie, J.A., 1999. Determinants of CD4 independence for a human immunodeficiency virus type 1 variant map outside regions required for coreceptor specificity. J. Virol. 73 (12), 10310–10319.

LaCasse, R.A., Follis, K.E., Moudgil, T., Trahey, M., Binley, J.M., Planelles, V., Zolla-Pazner, S., Nunberg, J.H., 1998. Coreceptor utilization by human immunodeficiency virus type 1 is not a primary determinant of neutralization sensitivity. J. Virol. 72 (3), 2491–2495.

Leavitt, M., Park, E.J., Sidorov, I.A., Dimitrov, D.S., Quinnan Jr., G.V., 2003. Concordant modulation of neutralization resistance and high infectivity of the primary human immunodeficiency virus type 1 MN strain and definition of a potential gp41 binding site in gp120. J. Virol. 77 (1), 560–570.

Lin, P.F., Blair, W., Wang, T., Spicer, T., Guo, Q., Zhou, N., Gong, Y.F., Wang, H.G., Rose, R., Yamanaka, G., Robinson, B., Li, C.B., Fridell, R., Deminie, C., Demers, G., Yang, Z., Zadjura, L., Meanwell, N., Colonno, R., 2003. A small molecule HIV-1 inhibitor that targets the HIV-1 envelope and inhibits CD4 receptor binding. Proc. Natl. Acad. Sci. U.S.A. 100 (19), 11013–11018.

Mascola, J.R., Lewis, M.G., Stiegler, G., Harris, D., Van Cott, T.C., Hayes, D., Louder, M.K., Brown, C.R., Sapan, C.V., Frankel, S.S., Lu, Y., Robb, M.L., Katinger, H., Birx, D.L., 1999. Protection of macaques against pathogenic simian/human immunodeficiency virus 89.6PD by passive transfer of neutralizing antibodies. J. Virol. 73 (5), 4009–4018.

Mascola, J.R., Stiegler, G., Van Cott, T.C., Katinger, H., Carpenter, C.B., Hanson, C.E., Beary, H., Hayes, D., Frankel, S.S., Birx, D.L., Lewis, M.G., 2000. Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. Nat. Med. 6 (2), 207–210.

Mo, H., Stamatatos, L., Ip, J.E., Barbas, C.F., Parren, P.W., Burton, D.R., Moore, J.P., Ho, D.D., 1997. Human immunodeficiency virus type 1 mutants that escape neutralization by human monoclonal antibody IgG1b12. J. Virol. 71 (9), 6869–6874.

Montefiori, D.C., Collman, R.G., Fouts, T.R., Zhou, J.Y., Bilska, M., Hoxie, J.A., Moore, J.P., Bolognesi, D.P., 1998. Evidence that antibody-mediated neutralization of human immunodeficiency virus type 1 by sera from infected individuals is independent of coreceptor usage. J. Virol. 72 (3), 1886–1893.

Moore, J.P., Cao, Y., Qing, L., Sattentau, Q.J., Pyati, J., Koduri, R., Robinson, J., Barbas, C.F., Burton, D.R., Ho, D.D., 1995. Primary isolates of human immunodeficiency virus type 1 are relatively resistant to neutralization by monoclonal antibodies to gp120, and their neutralization is not predicted by studies with monomeric gp120. J. Virol. 69 (1), 101–109.

Moore, J.P., Cao, Y., Leu, J., Qin, L., Korber, B., Ho, D.D., 1996. Inter- and intraclade neutralization of human immunodeficiency virus type: 1. genetic clades do not correspond to neutralization serotypes but partially correspond to gp120 antigenic serotypes. J. Virol. 70 (1), 427–444.

Muster, T., Steindl, F., Purtscher, M., Trkola, A., Klima, A., Himmler, G., Ruker, F., Katinger, H., 1993. A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. J. Virol. 67 (11), 6642–6647.

Muster, T., Guinea, R., Trkola, A., Purtscher, M., Klima, A., Steindl, F., Palese, P., Katinger, H., 1994. Cross-neutralizing activity against divergent human immunodeficiency virus type 1 isolates induced by the gp41 sequence ELDKWAS. J. Virol. 68 (6), 4031–4034.

Nyambi, P.N., Nkengasong, J., Lewi, P., Andries, K., Janssens, W., Fransen, K., Heyndrickx, L., Piot, P., van der Groen, G., 1996. Multivariate analysis of human immunodeficiency virus type 1 neutralization data. J. Virol. 70 (9), 6235–6243.

O'Brien, W.A., Mao, S.H., Cao, Y., Moore, J.P., 1994. Macrophage-tropic and T-cell line-adapted chimeric strains of human immunodeficiency virus type 1 differ in their susceptibilities to neutralization by soluble CD4 at different temperatures. J. Virol. 68 (8), 5264–5269.

Pantophlet, R., Ollmann, S.E., Poignard, P., Parren, P.W., Wilson, I.A., Burton, D.R., 2003. Fine mapping of the interaction of neutralizing and nonneutralizing monoclonal antibodies with the CD4 binding site of human immunodeficiency virus type 1 gp120. J. Virol. 77 (1), 642–658.

Park, E.J., Quinnan Jr., G.V., 1999. Both neutralization resistance and high infectivity phenotypes are caused by mutations of interacting residues in the human immunodeficiency virus type 1 gp41 leucine zipper and the gp120 receptor- and coreceptor-binding domains. J. Virol. 73 (7), 5707–5713.

Park, E.J., Vujcic, L.K., Anand, R., Theodore, T.S., Quinnan Jr., G.V., 1998. Mutations in both gp120 and gp41 are responsible for the broad neutralization resistance of variant human immunodeficiency virus type 1 MN to antibodies directed at V3 and non-V3 epitopes. J. Virol. 72 (9), 7099–7107.

Park, E.J., Gorny, M.K., Zolla-Pazner, S., Quinnan Jr., G.V., 2000. A global neutralization resistance phenotype of human immunodeficiency virus type 1 is determined by distinct mechanisms mediating enhanced infectivity and conformational change of the envelope complex. J. Virol. 74 (9), 4183–4191.

Pugach, P., Kuhmann, S.E., Taylor, J., Marozsan, A.J., Snyder, A., Ketas, T., Wolinsky, S.M., Korber, B.T., Moore, J.P., 2004. The prolonged culture of human immunodeficiency virus type 1 in primary lymphocytes increases its sensitivity to neutralization by soluble CD4. Virology 321 (1), 8–22.

Reeves, J.D., Miamidian, J.L., Biscone, M.J., Lee, F.-H., Ahmad, N., Pierson, T.C., Doms, R.W., 2004. Impact of mutations in the coreceptor binding site on human immunodeficiency virus type 1 fusion, infection, and entry inhibitor sensitivity. J. Virol. 78 (10), 5476–5485.

Reitz Jr., M.S., Wilson, C., Naugle, C., Gallo, R.C., Robert-Guroff, M., 1988. Generation of a neutralization-resistant variant of HIV-1 is due to selection for a point mutation in the envelope gene. Cell 54 (1), 57–63.

Rizzuto, C.D., Wyatt, R., Hernandez-Ramos, N., Sun, Y., Kwong, P.D., Hendrickson, W.A., Sodroski, J., 1998. A conserved HIV gp120 glycoprotein structure involved in chemokine receptor binding. Science 280 (5371), 1949–1953.

Roben, P., Moore, J.P., Thali, M., Sodroski, J., Barbas, C.F., Burton, D.R., 1994. Recognition properties of a panel of human recombinant Fab fragments to the CD4 binding site of gp120 that show differing abilities to neutralize human immunodeficiency virus type 1. J. Virol. 68 (8), 4821–4828.

Sanders, R.W., Korber, B., Lu, M., Berkhout, B., Moore, J.P., 2002. Mutational analyses and natural variability of the gp41 ectodomain. In: Kuiken, C.L., Foley, B., Freed, E., Hahn, B., Korber, B., Marx, P.A., McCutchan, F., Mellors, J.W., Wolinsky, S. (Eds.), HIV Sequence Compendium 2002. Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, Los Alamos, NM, pp. 43–68 (LA-UR 03-3564).

Sawyer, L.S., Wrin, M.T., Crawford-Miksza, L., Potts, B., Wu, Y., Weber, P.A., Alfonso, R.D., Hanson, C.V., 1994. Neutralization sensitivity of human immunodeficiency virus type 1 is determined in part by the cell in which the virus is propagated. J. Virol. 68 (3), 1342–1349.

Shibata, R., Igarashi, T., Haigwood, N., Buckler-White, A., Ogert, R., Ross, W., Willey, R., Cho, M.W., Martin, M.A., 1999. Neutralizing antibody directed against the HIV-1 envelope glycoprotein can completely block HIV-1/SIV chimeric virus infections of macaque monkeys. Nat. Med. 5 (2), 204–210.

Si, Z., Cayabyab, M., Sodroski, J., 2001. Envelope glycoprotein determinants of neutralization resistance in a simian-human immunodeficiency virus (SHIV-HXBc2P 3.2) derived by passage in monkeys. J. Virol. 75 (9), 4208–4218.

Thali, M., Olshevsky, U., Furman, C., Gabuzda, D., Li, J., Sodroski, J., 1991a. Effects of changes in gp120-CD4 binding affinity on human immunodeficiency virus type 1 envelope glycoprotein function and soluble CD4 sensitivity. J. Virol. 65 (9), 5007–5012.

Thali, M., Olshevsky, U., Furman, C., Gabuzda, D., Posner, M., Sodroski, J., 1991b. Characterization of a discontinuous human immunodeficiency virus type 1 gp120 epitope recognized by a broadly reactive neutralizing human monoclonal antibody. J. Virol. 65 (11), 6188–6193.

Thali, M., Moore, J.P., Furman, C., Charles, M., Ho, D.D., Robinson, J., Sodroski, J., 1993. Characterization of conserved human immunodeficiency virus type 1 gp120 neutralization epitopes exposed upon gp120-CD4 binding. J. Virol. 67 (7), 3978–3988.

Trkola, A., Pomales, A.B., Yuan, H., Korber, B., Maddon, P.J., Allaway, G.P., Katinger, H., Barbas, C.F., Burton, D.R., Ho, D.D., 1995. Cross-clade neutralization of primary isolates of human immunodeficiency virus type 1 by human monoclonal antibodies and tetrameric CD4-IgG. J. Virol. 69 (11), 6609–6617.

Trkola, A., Purtscher, M., Muster, T., Ballaun, C., Buchacher, A., Sullivan, N., Srinivasan, K., Sodroski, J., Moore, J.P., Katinger, H., 1996. Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. J. Virol. 70 (2), 1100–1108.

Trkola, A., Ketas, T., Kewalramani, V.N., Endorf, F., Binley, J.M., Katinger, H., Robinson, J., Littman, D.R., Moore, J.P., 1998. Neutralization sensitivity of human immunodeficiency virus type 1 primary isolates to antibodies and CD4-based reagents is independent of coreceptor usage. J. Virol. 72 (3), 1876–1885.

Veazey, R.S., Shattock, R.J., Pope, M., Kirijan, J.C., Jones, J., Hu, Q., Ketas, T., Marx, P.A., Klasse, P.J., Burton, D.R., Moore, J.P., 2003. Prevention of virus transmission to macaque monkeys by a vaginally applied monoclonal antibody to HIV-1 gp120. Nat. Med. 9 (3), 343–346.

Watkins, B.A., Buge, S., Aldrich, K., Davis, A.E., Robinson, J., Reitz Jr., M.S., Robert-Guroff, M., 1996. Resistance of human immunodeficiency virus type 1 to neutralization by natural antisera occurs through single amino acid substitutions that cause changes in antibody binding at multiple sites. J. Virol. 70 (12), 8431–8437.

Weber, J., Fenyo, E.M., Beddows, S., Kaleebu, P., Bjorndal, A., UNAIDS, 1996. Neutralization serotypes of human immunodeficiency virus type 1 field isolates are not predicted by genetic subtype. J. Virol. 70 (11), 7827–7832.

Wild, C., Dubay, J.W., Greenwell, T., Baird Jr., T., Oas, T.G., McDanal, C., Hunter, E., Matthews, T., 1994. Propensity for a leucine zipper-like domain of human immunodeficiency virus type 1 gp41 to form oligomers correlates with a role in virus-induced fusion rather than assembly of the glycoprotein complex. Proc. Natl. Acad. Sci. U.S.A. 91 (26), 12676–12680.

Wrin, T., Loh, T.P., Vennari, J.C., Schuitemaker, H., Nunberg, J.H., 1995. Adaptation to persistent growth in the H9 cell line renders a primary isolate of human immunodeficiency virus type 1 sensitive to neutralization by vaccine sera. J. Virol. 69 (1), 39–48.

Zwick, M.B., Labrijn, A.F., Wang, M., Spenlehauer, C., Saphire, E.O., Binley, J.M., Moore, J.P., Stiegler, G., Katinger, H., Burton, D.R., Parren, P.W., 2001. Broadly neutralizing antibodies targeted to the membrane-proximal external region of human immunodeficiency virus type 1 glycoprotein gp41. J. Virol. 75 (22), 10892–10905.