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Loss of Asparagine-Linked Glycosylation Sites in Variable Region 5 of Human Immunodeficiency Virus Type 1 Envelope Is Associated with Resistance to CD4 Antibody Ibalizumab▽

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Ibalizumab (formerly TNX-355) is a first-in-class, monoclonal antibody inhibitor of CD4-mediated human immunodeficiency type 1 (HIV-1) entry. Multiple clinical trials with HIV-infected patients have demonstrated the antiviral activity, safety, and tolerability of ibalizumab treatment. A 9-week phase Ib study adding ibalizumab monotherapy to failing drug regimens led to transient reductions in HIV viral loads and the evolution of HIV-1 variants with reduced susceptibility to ibalizumab. This report characterizes these variants by comparing the phenotypic susceptibilities and envelope (*env*) sequences of (i) paired baseline and on-treatment virus populations, (ii) individual *env* clones from selected paired samples, and (iii) *env* clones containing site-directed mutations. Viruses with reduced susceptibility to ibalizumab were found to exhibit reduced susceptibility to the anti-CD4 antibody RPA-T4. Conversely, susceptibility to soluble CD4, which targets the HIV-1 gp120 envelope protein, was enhanced. No changes in susceptibility to the fusion inhibitor enfuvirtide or the CCR5 antagonist maraviroc were observed. Functionally, viruses with reduced ibalizumab susceptibility also displayed high levels of infectivity relative to those of paired baseline viruses. Individual *env* clones exhibiting reduced ibalizumab susceptibility contained multiple amino acid changes in different regions relative to the paired baseline clones. In particular, clones with reduced susceptibility to ibalizumab contained fewer potential asparagine-linked glycosylation sites (PNGSs) in variable region 5 (V5) than did paired ibalizumab-susceptible clones. The reduction in ibalizumab susceptibility due to the loss of V5 PNGSs was confirmed by site-directed mutagenesis. Taken together, these findings provide important insights into resistance to this new class of antiretroviral drug.

Since the advent of highly active antiretroviral therapy (HAART), the number and variety of antiretroviral agents available to treat HIV-1 infections have increased steadily. Twenty-seven individual antiretroviral agents and five coformulated drug combinations representing five different mechanistic classes are currently approved for the treatment of HIV-1 infection (http://www.fda.gov/ForConsumers/byAudience/ForPatientAdvocates/HIVandAIDSActivities/ucm118915.htm). The five mechanistic classes include nucleoside reverse transcriptase inhibitors (NRTIs), nonnucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase inhibitors (INIs), and entry inhibitors (EIs), which to date include a fusion inhibitor and coreceptor antagonist. Treatment guidelines recommend the use of at least two, and preferably three, active agents in HAART regimens (21a). The selection of agents for a treatment regimen can be designed to balance the requirements for antiviral efficacy, safety, tolerability, and convenience. Intolerable side effects, negative drug-drug interactions, and complex dosing regimens can contribute to poor adherence, cessation of therapy, suboptimal viral suppression, and antiviral drug resistance. For these reasons, new agents with novel mechanisms of action that will combat resistance to existing therapies and exhibit fewer side effects or drug interactions are being pursued.

Ibalizumab (formerly TNX-355) is a novel antiretroviral agent in development for the treatment of HIV-1 infections. As a humanized IgG4 monoclonal antibody, ibalizumab blocks receptor-mediated virus entry by binding to extracellular domain 2 of the HIV-1 receptor CD4 with high affinity (K_d [dissociation constant] = 100 pM). Fine-mapping studies have demonstrated that this epitope is comprised of 5 amino acid residues in CD4 domain 2 and two residues in the C-terminal region of domain 1 (30). Located at the interface between domains 1 and 2 of the CD4 molecule, the ibalizumab binding epitope is on the opposite side of CD4 from the domain 1 binding sites that are required for major histocompatibility complex class II (MHCII) receptor binding and gp120 attachment. Ibalizumab exploits this unique mechanism to inhibit infection by a broad spectrum of HIV-1 isolates, including all major subtypes, irrespective of coreceptor tropism (5). In clinical studies, ibalizumab safely lowered plasma HIV-1 RNA levels in treatment-experienced patients at doses of up to 25 mg/kg of body weight following single-dose (15) and multiple-dose (11) administrations. Durable HIV-1 viral load reductions, accompanied by significant increases in $CD4^+$ T cell counts, were observed in a 48-week, randomized, double-blind, placebo-controlled phase II trial when ibalizumab was administered in combination with optimized background therapy (20a). Ibalizumab therapy was found to be well tolerated by all studies to date, with benign treatment-emergent adverse

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events, no significant safety concerns, and no evidence of immunosuppression. It is important that, while capable of inhibiting CD4-mediated HIV-1 entry, ibalizumab has not been shown to interfere with MHCII-mediated immune functions (25). This is consistent with the epitope map, which places the ibalizumab binding site on the side of CD4 opposite from that of the MHCII receptor. The emerging profile of ibalizumab as a safe and effective therapy for the treatment of HIV-1 infection is encouraging and supports further clinical development.

HIV-1 entry is an ordered, multistep process initiated by the binding of the gp120 subunit of the virus envelope protein to the cell surface receptor CD4. The attachment of gp120 to CD4 results in the exposure of sites on gp120 that mediate chemokine coreceptor binding, which in turn leads to the fusion of virus and cell membranes, a process mediated by the gp41 subunit of the envelope protein (4, 7). The binding of ibalizumab to domain 2 of CD4 does not interfere with the attachment of gp120 to domain 1 (20) but is thought to inhibit subsequent events in the entry process; however, the precise mechanism of inhibition has not been well characterized. Currently, there are two HIV-1 entry inhibitors approved for the treatment of HIV-1 infection: a fusion inhibitor, enfuvirtide (Fuzeon), and the CCR5 coreceptor antagonist maraviroc (Selzentry). Enfuvirtide competitively inhibits conformational changes in the HIV-1 gp41 protein and prevents membrane fusion (26). Maraviroc binds to CCR5 and interferes allosterically with gp120 engagement (40). Genetic determinants of resistance to enfuvirtide and maraviroc have been reported. Mutations in the enfuvirtide binding site within the heptad repeat 1 (HR1) region of gp41 dramatically reduce susceptibility to enfuvirtide (19, 26, 29, 37). Reductions in susceptibility to maraviroc have been associated with mutations in the V3 loop of gp120 that enable gp120 to bind to CCR5 in the presence of bound maraviroc (40). However, the primary route of maraviroc escape in the clinic appears to be the emergence of CXCR4-using variants (9, 39). Given our current understanding of these two entry inhibitors, it should not be overly surprising that there have been no reports of cross-resistance between maraviroc and enfuvirtide. This is consistent with the findings that the key determinants are distinct, both spatially (gp120 versus gp41) and functionally (coreceptor binding versus membrane fusion). However, sequence changes in gp120 have been shown to alter enfuvirtide susceptibility (6), whereas mutations in gp41 decreased maraviroc susceptibility in one recent study (1).

The initial phenotypic evaluation of ibalizumab escape variants indicated that reduced susceptibility to this agent is manifested by a reduction in the maximum percent inhibition (MPI) (11). This phenotype has also been reported for the CCR5 antagonists maraviroc, vicriviroc, and aplaviroc (18, 33, 35, 40) and is considered characteristic of "noncompetitive" inhibitors of HIV-1 entry. It has also been reported that ibalizumab-resistant isolates retain their dependence on the CD4-mediated entry pathway for infectivity (11). In this report, we have further characterized the phenotypic properties of ibalizumab-resistant patient viruses and have performed a clonal analysis of individual HIV-1 *env* variants from four patients. *env* sequences and the phenotypic susceptibilities of paired baseline and on-treatment virus populations were compared to identify specific genetic determinants of resistance. Escape mutations were verified by constructing and characterizing *env* clones containing or lacking specific site-directed mutations. Our observations further elucidate the unique mechanism of action for this novel agent and provide guidance for additional studies in support of the further clinical development of ibalizumab.

MATERIALS AND METHODS

Study subjects and samples. Fourteen study subjects enrolled in a phase Ib clinical evaluation of ibalizumab were included in the present study. Plasma-derived HIV-1 was isolated from all 14 subjects at baseline (day 0) and during treatment (week 9). *env* genes were amplified by using reverse transcription (RT)-PCR and cloned into an expression vector, generating *env* populations as previously described (41). Twelve individual *env* clones were obtained from each virus population for four subjects (subjects 004-2-04, 004-1-07, 004-1-02, and 004-2-06) at day 0 and week 9.

Site-directed mutagenesis. Glutamine (Q) was substituted for asparagine (N) in the V5 regions of patient envelope clones by site-directed mutagenesis (27) to disrupt the first (N460Q) and/or second (N464Q) potential N-linked glycosylation site (PNGS). PNGSs 1 and 2 refer to amino acid positions 460 to 462 and 464 to 466, respectively, of the HXB2 laboratory strain. Ibalizumab susceptibilities of parental and the various V5 PNGS knockout *env* clones were determined.

Susceptibility to entry inhibitors. Drug susceptibilities of patient virus populations, individual clones, and site-directed mutants were determined by using the PhenoSense HIV-1 Entry assay (19, 40). Susceptibilities to ibalizumab (TaiMed Biologics, Inc.) and other CD4 inhibitors, RPA-T4 (BD Pharmingen) and soluble CD4 (sCD4; Progenics), were determined by measuring the ability of pseudovirions to infect U87/CD4/CCR5/CXCR4 cells in the presence of serial dilutions of each inhibitor. The Wilcoxon signed-rank test was used to compare susceptibilities of day 0 and week 9 viruses to entry inhibitors. The coreceptor tropism of viruses was determined by measuring the ability of pseudovirions to infect U87/CD4/CCR5 and U87/CD4/CXCR4 cells in the absence and presence of coreceptor antagonists (41). Virus infectivity was examined by measuring luciferase production in infected target cells.

Sequence analyses of *env* clones. The nucleotide and derived amino acid sequences were determined by using conventional chain termination chemistry for 96 *env* clones derived from the day 0 and week 9 virus populations of four subjects (subjects 004-2-04, 004-1-07, 004-1-02, and 004-2-06).

RESULTS

Virologic rebound during ibalizumab add-on therapy is associated with the emergence of virus subpopulations exhibiting reduced susceptibility. In a phase Ib clinical trial, one of three multiple-dose regimens of ibalizumab was administered for 9 weeks to HIV-1-infected patients receiving failing or partially suppressive antiretroviral drug regimens (HIV viral load of >5,000 copies/ml). The anti-HIV-1 activity of ibalizumab was demonstrated by rapid reductions in HIV-1 viral loads and elevations in $CD4^+$ cell counts. Despite the continued administration of ibalizumab, reductions in viral load were transient and returned to baseline in most cases (11). To determine if changes in ibalizumab susceptibility were responsible for the observed rebounds in viral loads, we performed *in vitro* susceptibility assays on patient viruses obtained before and after ibalizumab treatment. Baseline (day 0) and on-treatment (week 9) samples from 14 study subjects were available for evaluation. Pseudovirions expressing the day 0 virus *env* genes were susceptible to ibalizumab. In contrast, pseudovirions expressing week 9 virus *env* genes exhibited notable reductions in susceptibility to ibalizumab, manifested as reductions in the maximum percent inhibition (MPI) (Fig. 1A). The ibalizumab MPI ranged from 89% to 99% (median, 97%) for day 0 viruses, versus 33% to 83% (median, 64%) for week 9 viruses ($P < 0.0001$ by Wilcoxon signed-rank test).

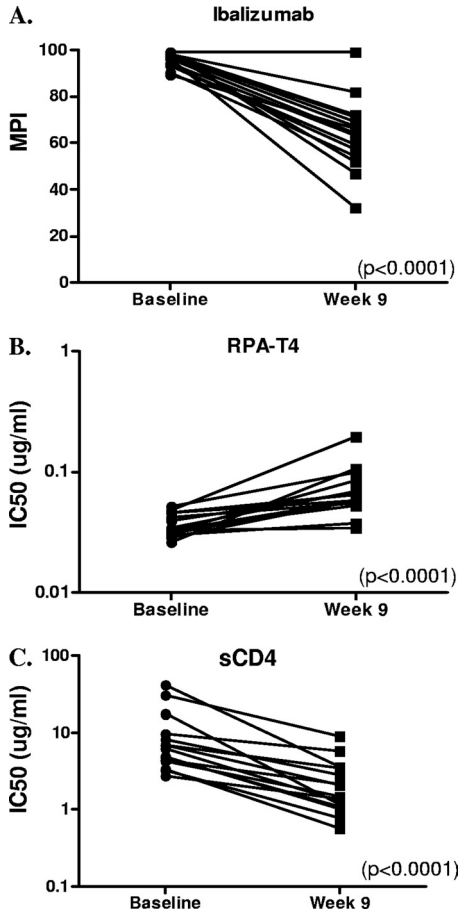

FIG. 1. Ibalizumab susceptibility of viruses from 14 subjects at day 0 and week 9 of treatment. Susceptibility was measured by using the PhenoSense Entry assay and is represented as the maximum percent inhibition (MPI) of ibalizumab (A) or fold change in the IC_{50} of the CD4 antibody RPA-T4 (B) and sCD4 (C).

Reductions in ibalizumab susceptibility are associated with alterations in susceptibility to attachment (CD4-gp120) inhibitors. To determine whether reductions in ibalizumab susceptibility altered susceptibility to HIV attachment inhibitors, we evaluated the susceptibilities of day 0 and week 9 viruses to a CD4-targeted antibody (RPA-T4) and soluble CD4 (sCD4). Unlike ibalizumab, these two inhibitors competitively block HIV-1 attachment by binding to either the gp120 binding site of CD4 (RPA-T4) or the CD4 binding site of gp120 (sCD4). Susceptibilities of paired day 0 and week 9 viruses from each subject to RPA-T4 and sCD4 are shown in Fig. 1B and C. Compared to the paired day 0 virus, each week 9 virus exhibited reduced susceptibility to RPA-T4. The median 50% inhibitory concentrations (IC_{50}s) for RPA-T4 were 0.03 μg/ml (range, 0.02 to 0.05 μg/ml) for day 0 viruses and 0.06 μg/ml (range, 0.03 to 0.20 μg/ml) for week 9 viruses. Although not large, these reductions in RPA-T4 susceptibility were statistically significant ($P < 0.0001$ by Wilcoxon signed-rank test). Conversely, week 9 viruses were significantly ($P < 0.0001$ by Wilcoxon signed-rank test) more susceptible to inhibition by sCD4 (median IC_{50}, 1.47 μg/ml; range, 0.57 to 8.96 μg/ml) than the paired day 0 viruses (median IC_{50}, 6.24 μg/ml; range, 2.70 to 41.68 μg/ml). Consistent with competitive mechanisms of inhibition, alterations in RPA-T4 and sCD4 susceptibilities were associated with changes in IC_{50}s rather than changes in MPIs at saturating drug concentrations. In contrast to RPA-T4 and sCD4, no differences in enfuvirtide susceptibility or maraviroc susceptibility were observed between paired day 0 and week 9 ibalizumab-treated viruses (data not shown).

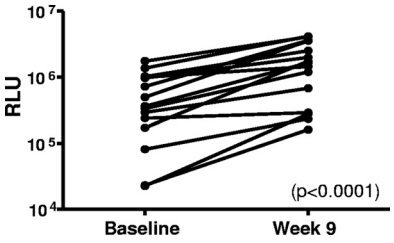

FIG. 2. Env-mediated infectivity of baseline and ibalizumab-treated viruses. Env-mediated infectivity was assessed by measuring the luciferase activity of pseudovirions expressing the Env proteins from 14 subjects at day 0 and week 9.

Viruses with reduced ibalizumab susceptibility exhibit increased infectivity. Previous studies demonstrated that alterations in gp120-CD4 interactions can impact viral infectivity and membrane fusion (8, 22, 31, 32). To investigate whether reductions in ibalizumab susceptibility affect these Env-mediated functions, we assessed the *in vitro* infectivities of paired day 0 and week 9 virus isolates by comparing reporter gene expression levels in the HIV-1 entry assay. Based upon luciferase activity (relative light units [RLU]), the infectivity of the week 9 virus isolates was on average 3-fold higher than those of the paired day 0 viruses (mean RLU of 1,824,301 and 587,050, respectively) (Fig. 2).

Composition of ibalizumab-susceptible and -resistant virus populations. To determine whether the degree of ibalizumab inhibition (MPI) reflects different proportional mixtures of ibalizumab-susceptible and -resistant variants or a common phenotype of the dominant variants, we isolated 12 individual *env* clones from the day 0 and week 9 viruses of four subjects (subjects 004-2-04, 004-1-07, 004-1-02, and 004-2-06). Ibalizumab susceptibilities for the four paired virus populations and all clones are summarized in Table 1, and the ibalizumab inhibition curves of four paired day 0 and week 9 viruses are shown in Fig. 3. Both the starting populations and the individual clones from week 9 samples exhibited decreased susceptibility to ibalizumab (i.e., lower MPI) relative to the susceptibilities of the corresponding day 0 viruses (median ibalizumab MPI of 99% for 48 day 0 clones versus 60.5% for 48 week 9 clones; $P < 0.0001$ by Wilcoxon signed-rank test). Overall, the inhibition of the majority of day 0 clones (45/48 clones) exceeded 85% (MPI), whereas the inhibition of the majority of clones from week 9 samples (42/48 clones) did not reach 85% (MPI). The heterogeneity of inhibitory profiles of the *env* clones indicates that the MPI observed for each *env* population reflects a mixture of variants that are differentially inhibited by ibalizumab. These observations indicate that ibalizumab resistance is associated with the increased prevalence of variants that are less efficiently inhibited than the pretreatment variants (Table 1). Consistent with our observations of *env* populations (Fig. 1), reductions in the ibalizumab susceptibility of *env*

TABLE 1. Analysis of *env* clones from four study subjects at day 0 and week 9 of ibalizumab treatment[a]

Subject (identification)	Time point	Ibalizumab MPI of the *env* population	Median ibalizumab MPI of *env* clones (range)	No. of clones based on coreceptor tropism		
				R5	X4	Dual
1 (004-2-04)	Day 0	99	98 (45–100)	12		
	Wk 9	73	22 (<1–98)	12		
2 (004-01-07)	Day 0	94	100 (93–100)	2	1	9
	Wk 9	61	68 (37–86)		2	10
3 (004-01-02)	Day 0	93	95 (85–99)		3	9
	Wk 9	60	49 (30–72)	2	7	3
4 (004-02-06)	Day 0	98	99 (95–100)	10	2	
	Wk 9	67	73 (62–90)	10	2	

[a] A total of 96 *env* clones from subjects 004-2-04, 004-1-07, 004-1-02, and 004-2-06 were analyzed. Twenty-four clones from each subject were evaluated (12 clones from day 0 and 12 clones from week 9 virus populations).

clones (Fig. 4A) correlated directly with reductions in RPA-T4 susceptibility (Fig. 4B) and inversely with increases in sCD4 susceptibility (Fig. 4C).

Based on the phenotypic analysis of *env* clones, three of four subjects (subjects 004-1-07, 004-1-02, and 004-2-06) were infected with mixed-tropic virus populations comprised of R5-, X4-, and/or dual-tropic clones, while the remaining subject (subject 004-42-04) harbored an R5-tropic virus population (Table 1). Although limited in sample size, a comparison of *env* tropisms at day 0 and week 9 indicates that coreceptor tropism does not directly influence ibalizumab susceptibility or the development of ibalizumab resistance.

***env* sequences of ibalizumab-resistant variants.** In order to investigate the genetic determinants and underlying mechanism of ibalizumab resistance, we determined the nucleotide sequences of the full-length (gp160) *env* of all 96 clones from the four selected subjects and explored their relatedness by assembling phylogenetic trees. These analyses revealed that day 0 and week 9 *env* clones from each subject clustered tightly in branches that clearly distinguished each subject from the others. Notably, day 0 and week 9 clones of each pair were not always temporally segregated within each patient cluster, which would support a model in which ibalizumab resistance most likely occurs by the outgrowth of minor preexisting variants rather than by the acquisition of *de novo* mutations. Mutations distinguishing day 0 and week 9 clones were distributed throughout the *env* gene, especially in the gp120 region. Notably, no amino acid differences were observed for the CD4 binding site of gp120 (16) between day 0 and week 9 clones for all four subjects analyzed.

Both the V1/V2 length and the number of potential N-linked glycosylation sites (PNGSs) within gp120 have been reported to affect sensitivity to sCD4, CD4 binding site exposure, and/or fusogenicity (14, 23, 31). A comparison of the lengths and numbers of PNGSs for each of the variable regions of day 0 and corresponding week 9 clones is presented in Table 2. Overall, the paired day 0 and week 9 clones from all four subjects exhibited little or no consistent change in the length of either V1/V2 or the other variable regions. Similarly, the number of PNGSs within each of the variable regions exhibited little or no consistent change between paired day 0 and week 9 clones, with the notable exception of the V5 region. Within V5, week 9 clones contained fewer PNGSs than the paired day 0 clones for all four subjects (Table 2).

Diminished ibalizumab inhibition is conferred by reductions in V5 N-linked glycosylation sites. An inspection of the PNGS in V5 and ibalizumab susceptibility for individual clones revealed a strong correlation between the number of V5 glycosylation sites and ibalizumab susceptibility (Table 3). In general, *env* clones with two PNGSs in V5 were efficiently inhibited by ibalizumab, while *env* clones with a single V5 PNGS exhibited reduced susceptibility, and *env* clones lacking V5

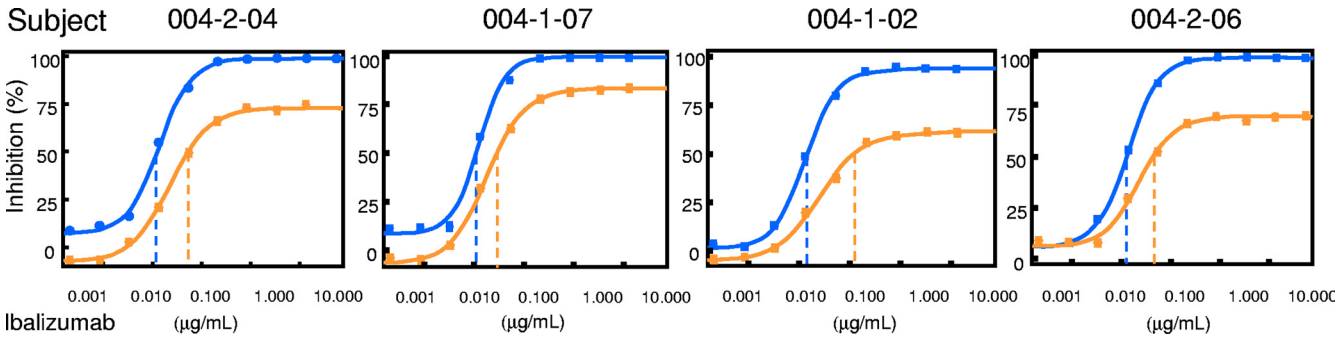

FIG. 3. Ibalizumab susceptibilities of baseline and ibalizumab-treated viruses from four representative subjects. Ibalizumab inhibition of pseudovirions expressing the Env proteins from subjects 004-2-04, 004-1-07, 004-1-02, and 004-2-06 was measured for the paired day 0 (blue) and week 9 (orange) viruses.

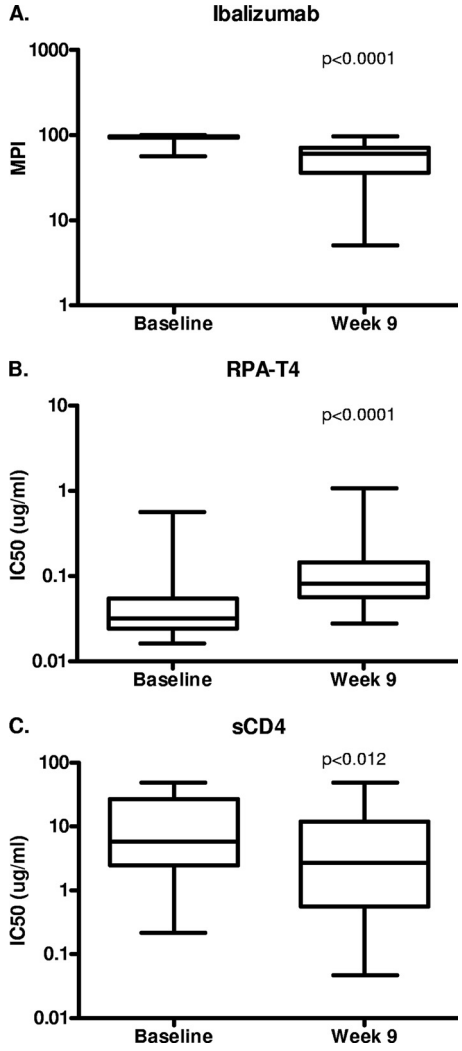

FIG. 4. Phenotypic susceptibilities of *env* clones from four representative subjects at day 0 and week 9. The susceptibilities of 96 clones from subjects 004-2-04, 004-1-07, 004-1-02, and 004-2-06 were tested by using the PhenoSense Entry assay and are represented as maximum percent inhibition (MPI) of ibalizumab (A) or fold changes in the IC_{50}s of the CD4 antibody RPA-T4 (B) and sCD4 (C).

PNGSs were resistant to ibalizumab (median MPIs, 99%, 71%, and 40%, respectively). All 48 clones from the four day 0 samples contained one or two PNGSs in the V5 region; the most conserved positions were at amino acids 460 to 462 (site 1) and 464 to 466 (site 2) relative to the HXB2 sequence (Table 4). For three subjects (subjects 004-2-04, 004-1-07, and 004-2-06), the majority of day 0 clones (11/12, 9/12, and 12/12 clones, respectively) possessed two PNGSs, while day 0 clones from the remaining subject (subject 004-1-02) contained two sites for 5/12 clones and a single site (site 2) for 7/12 clones. The majority of week 9 clones possessed one fewer V5 glycosylation site than the corresponding day 0 clones. For the three subjects with two V5 PNGSs in most of the day 0 clones (subjects 004-2-04, 004-1-07, and 004-2-06), the majority of the week 9 clones (30/36 clones) possessed a single PNGS. For the remaining subject (subject 004-1-02) with a single site in 7/12 day 0 clones, 8/12 week 9 clones lacked V5 PNGSs altogether. Taken together, these data suggest that efficient ibalizumab inhibition is dependent on N-linked glycosylation in the V5 region. Furthermore, a clear spatial pattern in the V5 PNGS was observed. Of the 35 week 9 clones that contained just one V5 PNGS, all of them retained the site 2 PNGS. This indicates that the site 1 PNGS is eliminated disproportionately in viruses with reduced susceptibility to ibalizumab.

To verify the role of V5 PNGSs on ibalizumab susceptibility, site-directed mutagenesis was used to selectively delete one or both PNGS (Δsite 1, Δsite 2, Δsite 1 and 2) from an ibalizumab-susceptible *env* clone (with an MPI of 100%). The removal of the site 2 PNGS did not alter ibalizumab susceptibility (Fig. 5), while the removal of the site 1 PNGS clearly reduced ibalizumab susceptibility (MPI of 65%). The removal of both sites further reduced ibalizumab susceptibility (MPI of 25%). No other changes in this *env* clone were required to significantly alter ibalizumab susceptibility. Additional site-directed mutagenesis analyses have been performed by another laboratory using a different *env* backbone, and concordant results were observed, confirming the association of V5 PNGSs with ibalizumab susceptibility (David Ho, personal communication). These results are consistent with the observed PNGS patterns of day 0 and week 9 *env* clones described above and strengthen the association between V5 glycosylation and ibalizumab susceptibility. In addition, the site-directed mutagenesis data are consistent with a stronger association between the loss of PNGS 1 and the reduced ibalizumab susceptibility observed for patient viruses. An important and notable distinction between the ibalizumab-resistant variants that emerge *in vivo* and those created by site-directed mutagenesis is that susceptibility to sCD4 was not affected by the site-directed elimination of V5 PNGSs (data not shown). This observation suggests that V5 PNGSs have a specific effect on ibalizumab susceptibility that is segregated from sCD4 susceptibility.

It is important that clones containing the same number of V5 PNGSs can exhibit different susceptibilities to ibalizumab. For example, clones with two or more V5 PNGSs exhibited a broad range of ibalizumab susceptibilities (MPI of 13 to 100%), as did clones with one V5 PNGS (MPI of <0 to 100%) (Table 3). Furthermore, three of the week 9 clones from patient 004-2-04 bearing two V5 PNGSs and identical V5 amino acid sequences demonstrated dramatically different susceptibilities to ibalizumab, ranging from 13 to 98% MPI (Table 4). These observations illustrate the role of other *env* determinants in ibalizumab susceptibility and the need for further studies to identify these determinants and characterize their effects.

DISCUSSION

Viruses with reduced susceptibility to ibalizumab were identified in patients who experienced a rebound in the HIV-1 viral load after the addition of ibalizumab to failing antiretroviral drug regimens. The susceptibilities of these viruses and their paired baseline isolates to ibalizumab and other HIV-1 entry inhibitors were evaluated by using cell-based infectivity assays. Compared to matching baseline viruses, the infectivity of viruses isolated after viral load rebound was incompletely inhibited by ibalizumab. This phenotypic profile most likely reflects the ability of ibalizumab-resistant envelope proteins to mediate

TABLE 2. Lengths and numbers of glycosylation sites in Env variable regions of day 0 and week 9 *env* clones

Subject (identification)	Time point[c]	Length of amino acid sequence in *env* region[a]						No. of PNGSs in *env* region[a]					
		gp160	V1	V2	V3	V4	V5	gp160	V1	V2	V3	V4	V5[b]
1 (004-2-04)	Day 0	835.3	38.0	39.0	35.0	29.8	12.5	30.9	5.1	2.0	1.0	4.9	2.0
	Wk 9	835.3	38.4	39.0	35.0	29.5	12.3	29.9	5.1	2.0	1.0	4.7	1.3
	Day 0/wk 9	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.1	1.5
2 (004-1-07)	Day 0	864.1	38.2	41.8	34.5	34.0	14.3	32.4	5.9	2.5	0.5	5.4	1.8
	Wk 9	844.8	32.3	45.0	34.0	34.2	14.1	31.7	5.0	3.0	0.0	5.9	1.0
	Day 0/wk 9	1.0	1.2	0.9	1.0	1.0	1.0	1.0	1.2	0.8	ND	0.9	1.8
3 (004-1-02)	Day 0	867.3	37.4	46.0	35.0	31.0	15.0	33.3	4.5	4.0	0.3	5.0	1.4
	Wk 9	864.7	36.3	46.0	35.0	31.0	14.3	30.0	4.0	3.8	0.3	4.9	0.3
	Day 0/wk 9	1.0	1.0	1.0	1.0	1.0	1.0	1.1	1.1	1.0	1.0	1.0	4.3
4 (004-2-06)	Day 0	842.8	29.1	43.0	35.0	30.0	17.0	29.7	4.0	3.0	1.7	1.1	2.1
	Wk 9	858.7	28.0	43.0	35.0	30.0	16.7	29.1	4.0	3.0	1.7	1.1	1.0
	Day 0/wk 9	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.1
All clones from 4 subjects	Day 0	852.4	35.7	42.5	34.9	31.2	14.7	31.6	4.9	2.9	0.9	4.1	1.8
	Wk 9	850.8	33.8	43.3	34.8	31.2	14.4	30.2	4.5	3.0	0.8	4.1	0.9
	Day 0/wk 9	1.0	1.1	1.0	1.0	1.0	1.0	1.0	1.1	1.0	1.2	1.0	2.0

[a] A total of 96 *env* clones from four subjects (subjects 004-2-04, 004-1-07, 004-1-02, and 004-2-06) were analyzed (12 clones from day 0 and 12 clones from week 9 for each virus population). The average amino acid lengths and the numbers of potential N-linked glycosylation sites are shown. ND, not determined.
[b] Week 9 clones have fewer V5 PNGSs than their paired day 0 clones. The ratios of day 0 to week 9 V5 PNGSs are shaded.
[c] Day 0/wk 9 indicates the ratio of the day 0 value to the week 9 value.

CD4-dependent HIV-1 entry in the presence of bound ibalizumab (11). A similar inhibitory profile was reported previously for viruses that have acquired reduced susceptibility to noncompetitive CCR5 antagonists, including maraviroc (40), vicriviroc (18, 35), and aplaviroc (12, 33). In these instances, resistant viruses regained the ability to bind CCR5 despite the presence of a bound antagonist (24, 33, 40). Similarly, ibalizumab is also a noncompetitive inhibitor which does not block gp120 binding to CD4 (20) but rather is thought to prevent downstream events required for efficient HIV-1 entry. Such events might include conformational changes in gp120 (28) and/or CD4 (2) that are triggered by CD4-gp120 binding, coreceptor engagement (34, 42), or subsequent events leading to membrane fusion. Whether ibalizumab inhibition occurs by direct or indirect interference in such events is currently unresolved.

In this study we demonstrated that viruses with reduced susceptibility to ibalizumab also exhibited reduced susceptibility to the anti-CD4 monoclonal antibody RPA-T4. This observation suggests that the competitive inhibition of CD4-gp120 binding by RPA-T4 is less efficient with ibalizumab-resistant viruses and is consistent with the previously reported behavior of viruses selected *in vitro* for reduced susceptibility to the monoclonal antibody 5A8, the murine progenitor of ibalizumab (13). In addition, our studies revealed a striking inverse relationship between the susceptibilities of viruses to ibalizumab and sCD4, which targets the CD4 binding site of gp120; that is, reductions in susceptibility to ibalizumab are accompanied by increases in susceptibility to sCD4. Interestingly, viruses with reduced ibalizumab susceptibility (week 9) displayed measurably higher levels of *env*-mediated infectivity *in vitro* than did the paired ibalizumab-susceptible (day 0) viruses. The observed increases in virus infectivity are consistent with increased susceptibility to sCD4 if one assumes more efficient interactions between viral gp120 and cell surface CD4. This increased susceptibility to sCD4 is not associated with a detectable increase in susceptibility to antibody neutralization, since week 9 viruses were not more susceptible than their paired day 0 viruses to the broadly neutralizing monoclonal antibodies b12, 2G12, and 4E10 or polyclonal HIV-1 plasma antibodies (data not shown). Further studies are needed to evaluate the impact of viral replication of ibalizumab-resistant viruses *in vivo*.

TABLE 3. The presence of V5 N-linked glycosylation sites is associated with ibalizumab susceptibility

No. of V5 PNGSs[a]	No. of clones	MPI of ibalizumab		
		Range	Median	*P* value[b]
2	42	13–100	99	
1	45	<1–100	71	<0.0001
0	9	30–78	40	<0.0001

[a] Ninety-six *env* clones from four subjects (subjects 004-2-04, 004-1-07, 004-1-02, and 004-2-06) were analyzed (12 clones from day 0 and 12 clones from week 9 for each patient virus population). One clone with 3 PNGSs (subject 004-2-04 baseline) was included together with clones with 2 PNGSs for analysis.
[b] The Wilcoxon signed-rank test was used to compare ibalizumab MPIs between clones with 1 or 0 PNGSs versus 2 PNGSs.

To begin to experimentally delineate the genetic determinants of ibalizumab resistance, an in-depth analysis of *env* clones isolated from four paired baseline and on-treatment virus populations was conducted. The phenotypic properties of the 96 individual clones consistently mirrored the pooled *env* sequence populations sampled at day 0 and week 9 with respect to susceptibility to ibalizumab and other entry inhibitors. Phylogenetic analyses of day 0 and week 9 clones from each patient suggested that ibalizumab-resistant variants may emerge by the outgrowth of preexisting minority variants present in the baseline virus population; however, this and other

TABLE 4. Ibalizumab susceptibility and V5 sequences of day 0 and week 9 *env* clones

Subject (identification) (time point)	No. of clones	Avg ibalizumab MPI (range)	V5 sequence[a]	No. of V5 PNGSs[c]			V5 length (aa)[d]
				Total	Positions 460–462 (site 1)	Positions 464–466 (site 2)	
1 (004-2-04) (day 0)	3	99 (99–100)	GGANNS--INETFRPG	2	1	1	14
	7	94 (69–100)	GGVNNT---NETFRPG	2	1	1	13
	1	67	GGSHNNTTTNETFRPG[b]	3	1	1	16
	1	45	GGVNNA---NETFRPG	1	0	1	13
1 (004-2-04) (wk 9)	1	96	GGVNST---NETFRPG	2	1	1	13
	1	63	GGVTNT---NETFRPG	1	0	1	14
	3	52 (13–98)	GGVNNT---NETFRPG	2	1	1	14
	1	31	GGVDST---NETFRPG	1	0	1	14
	4	4 (−18–50)	GGANNN--INETFRPG	1	0	1	14
	2	−22 (−38–−6)	GGVNNI---NETFRPG	1	0	1	14
2 (004-1-07) (day 0)	2	100 (100–100)	G-VNTTKNTEIFRPG	1	1	0	14
	4	100 (100–100)	GNTTTTNNTEIFRPG	2	1	1	15
	1	100	G-NTTMNTTEIFRPG	2	1	1	14
	2	100 (99–100)	G-NTTTNNTEIFRPG	2	1	1	14
	2	100 (99–100)	G-NTTANNTEIFRPG	2	1	1	14
	1	93	G-NTNTNNTEIFRPG	1	0	1	14
2 (004-1-07) (wk 9)	1	86	GNTPTTNNTEIFRPG	1	0	1	15
	2	59 (49–69)	G-NTDTNNTEIFRPG	1	0	1	14
	9	65 (37–86)	G-NTNTNNTEIFRPG	1	0	1	14
3 (004-1-02) (day 0)	3	99 (99–99)	GNNNGTNETEIFRPE	2	1	1	15
	2	98 (96–99)	GNNNGSNETEIFRPG	2	1	1	15
	7	92 (85–96)	GNNNGNNETEIFRPG	1	0	1	15
3 (004-1-02) (wk 9)	4	63 (59–72)	GNNNGNNETEIFRPG	1	0	1	15
	2	48 (34–63)	GNNNGNHETEIFRPG	0	0	0	15
	1	56	GNNNGNSETEIFRPG	0	0	0	15
	4	40 (38–43)	GNNNGNNE--IFRPG	0	0	0	13
	1	30	GNNNNNKETEIFRPG	0	0	0	15
4 (004-2-06) (day 0)	1	100	GNNNNSIANKTETFRPG	2	1	1	17
	1	99	GNNNNSSTNKTETFRPG	2	1	1	17
	1	99	GNNNNSIINKTETFRPG	2	1	1	17
	5	98 (96–99)	GNNNNSITNKTETFRPG	2	1	1	17
	4	97 (95–99)	GNNNDSSTNKTEIFRPG	2	1	1	17
4 (004-2-06) (wk 9)	3	79 (71–90)	GNNNNRNTNKTETFRPG	1	0	1	17
	5	71 (64–86)	GNNDNSNTNKTETFRPG	1	0	1	17
	1	86	GNNNDSSKNKTETFRPG	2	1	1	17
	1	78	GNNND----KTEIFRPG	0	0	0	13
	1	72	GNNYNSGTNKTETFRPG	1	0	1	17
	1	62	GNNNNGITNKTETFRPG	1	0	1	17

[a] The numbers of clones with the indicated V5 sequences are shown. V5 PNGSs at site 1 and site 2 are underlined.
[b] One clone with 3 PNGSs (subject 1 [004-2-04] baseline) is indicated.
[c] The total number of V5 PNGSs and the numbers of PNGSs at site 1 and 2 are indicated.
[d] aa, amino acids.

potential mechanisms will require more extensive evaluation. A careful scrutiny of *env* sequences identified a common mutation pattern associated with reduced susceptibility to ibalizumab. Specifically, mutations that disrupt PNGSs in the V5 region of gp120 are key determinants of ibalizumab resistance, which was further substantiated by site-directed mutagenesis experiments. Among the two highly conserved PNGSs in V5 (site 1 and site 2), site 1 was most often absent in ibalizumab-resistant envelope proteins. Note that exceptions were observed, i.e., ibalizumab-resistant variants possessing two V5 PNGSs. These data suggest that additional genetic determinants can confer reductions in susceptibility to ibalizumab; however, their identification and characterization will require further study.

HIV Env contains numerous N-linked carbohydrates, some of which are important determinants of antibody neutralization (17, 36, 38). The elimination of PNGS and reduced ibalizumab susceptibility are consistent with the notion that these mutations restore an essential CD4-dependent conformational change(s) in gp120 despite drug binding. The V5 loop is situated on the outer domain of gp120 and comprises a portion of the so-called "glycan shield," which is thought to reduce the immunogenicity of this exposed facet of the gp120 protein structure (38, 43). An additional perspective on the mechanism

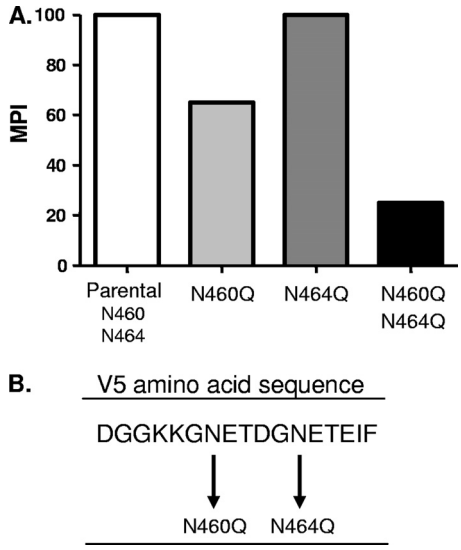

FIG. 5. Effect of V5 N-linked glycosylation sites on ibalizumab susceptibility. (A) Ibalizumab susceptibilities of a parental *env* clone containing site 1 (positions 460 to 462) and site 2 (position 464 to 466) PNGSs and PNGS 1 and 2 knockout clones. (B) V5 amino acid sequences of the parental *env* clone and the PNGS knockout clones.

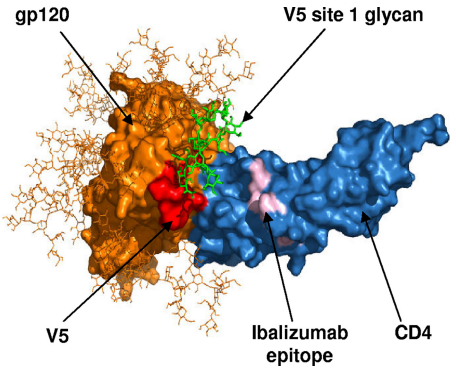

FIG. 6. V5 glycans are situated in close proximity to the gp120-CD4 interface. The interface of the gp120 core structure (orange) with CD4 (blue) is adjacent to the V5 region (red) and the V5 site 1 glycan located at amino acid position 460 (green). gp120 glycans were modeled by using the Glycosylate software package (courtesy of Jiang Zhu). Two positions in domain 1 (E77 and S79) and three positions in domain 2 (P121, P122, and Q163) of CD4 that are essential for ibalizumab binding (30) are highlighted (pink).

of ibalizumab resistance may be gained from studies of the mechanism of sCD4 inhibition. sCD4 inhibits HIV-1 entry either by the competitive inhibition of gp120 binding or by promoting irreversible conformational changes leading to an inactivation of infectivity. Differences in the affinity of gp120 for sCD4 do not account for reductions in susceptibility to ibalizumab (3, 20, 21) or the murine precursor, mu5A8 (13). Conformational changes in gp120 triggered by sCD4 are transient and render gp120 competent, albeit briefly, for receptor-mediated virus entry (10). These same changes are also likely involved in the host cell CD4-mediated activation of gp120, although it is unclear how this differs functionally from activation by sCD4. Thus, it is reasonable to postulate that ibalizumab escape variants can bypass this requirement by enhancing the ability to trigger CD4-induced conformational changes or, less likely, by developing an alternative mechanism of gp120 activation, since the latter is inconsistent with the observed CD4 dependency of ibalizumab-resistant isolates. Alternatively, ibalizumab may block downstream events such as coreceptor binding or other conformational changes that are required for membrane fusion.

Analysis of 1,322 nonredundant *env* sequences in the Los Alamos National Laboratory HIV Database demonstrated that the V5 sequences of 97.9% and 56.3% of subtype B sequences contain one and two PNGSs, respectively. This conservation of PNGS is consistent with a determinant role in broad ibalizumab susceptibility. To further explore the role of V5 glycosylation in ibalizumab susceptibility, we modeled the interaction between gp120 and CD4 using the structure reported under Protein Data Bank (PDB) accession number 1GC1 and PNGSs using the Glycosylate software program (courtesy of Jiang Zhu). Based on this model, the V5 site 1 glycan is situated in close proximity to the CD4-gp120 interface (Fig. 6). This proximity elicits several potential mechanisms of ibalizumab activity and escape. In one instance, CD4-ibalizumab binding may result in a steric clash between gp120 carbohydrate moieties and CD4 that prevents CD4-gp120 binding. Such a constraint may be removed by the elimination of V5 carbohydrate side chains. However, this mechanism is inconsistent with data from previous studies demonstrating the ability of gp120 to bind CD4 in the presence of bound ibalizumab (20, 28). Alternatively, the loss of V5 glycosylation may increase the binding affinity of gp120 for CD4 in the presence of ibalizumab. However, this mechanism is inconsistent with data from previous studies demonstrating comparable CD4-gp120 binding affinities for ibalizumab-susceptible and -resistant variants (3, 20, 21) and the murine antibody precursor 5A8 (13). Based on existing observations, the most probable explanation for ibalizumab escape is the ability of ibalizumab-resistant variants to facilitate CD4-induced conformational changes in the CD4-gp120 complex, which enable coreceptor engagement despite bound ibalizumab. Further investigation will be required to confirm this hypothesis or determine whether the inhibitory mechanism involves events further downstream in the HIV entry process. Either way, the reestablishment of requisite conformational changes may be enabled by the elimination of steric hindrances imposed by one or more V5 glycosylation moieties.

From a clinical standpoint, the lack of detectable cross-resistance between ibalizumab and other entry inhibitors such as maraviroc and enfuvirtide suggests that there exists a potential to prescribe ibalizumab irrespective of prior entry inhibitor therapy or coreceptor tropism. Consistent with the observed lack of cross-resistance, critical determinants of maraviroc and enfuvirtide resistance have been mapped to the V3 region and gp41, respectively, and do not appear to overlap with the determinants of ibalizumab resistance reported in this study. *In vitro* drug combination studies have demonstrated synergistic antiviral activities for ibalizumab and enfuvirtide (44) and additive-to-synergistic activity for ibalizumab and maraviroc (S. P. Weinheimer et al., unpublished data), providing further support for ibalizumab as a potentially valuable addition to antiretroviral drug regimens. However, more stud-

ies are needed to further evaluate the potential for cross-resistance between ibalizumab and other entry inhibitors and the effects of combination therapy in the clinical setting.

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