Emergence of Autologous Neutralization-Resistant Variants from Preexisting Human Immunodeficiency Virus (HIV) Quasi Species during Virus Rebound in HIV Type 1–Infected Patients Undergoing Highly Active Antiretroviral Therapy

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The role of neutralizing antibodies (NAbs) during virus rebound in human immunodeficiency virus type 1 (HIV-1)–infected patients undergoing highly active antiretroviral therapy is poorly understood. Three patients in this study had NAbs to preexisting autologous HIV-1 and an episode of virus rebound after a prolonged period of virus suppression. To investigate the influence of NAbs on virus evolution, envelope genotypes of preexisting and rebound viruses were examined. Phylogenetic analysis of env (V1–V5) sequences indicated that rebound viruses had evolved from or preexisted in baseline populations. By use of envelope pseudotype viruses, rebound viruses were found to be significantly resistant to neutralization by autologous antibody in all 3 patients, indicating that rebound viruses were selected by NAbs. The site responsible for conferring neutralization resistance against autologous antibody was identified in the upstream C3 region in 2 of 3 patients.

Neutralizing antibody (NAb) appears to be an important component in the protective immune response against human immunodeficiency virus type 1 (HIV-1) infection [1]. Broad NAb responses have been observed in long-term nonprogressors (LTNPs) [2], and LTNPs have significantly higher NAb titers than rapid progressors [1]. Some persons have NAbs against autologous isolates [3–5], but the emergence of escaped viruses with a reduced sensitivity to neutralization by autologous sera has also been observed [5]. In many studies, neutralization escape mutants have been sought by culturing primary neutralization-sensitive HIV-1 strains in the presence of certain NAbs in vitro [5–9] or in animal model systems by use of recombinant viruses [10–15]. Others have reported on conformational changes in gp120 effected by antibody-binding epitopes, as determined by neutralizing monoclonal antibody (MAb)–binding assay and antibody competition analysis [11, 16, 17]. However, there is little information on the influence of host factors, including NAbs, on virus evolution during highly active antiretroviral therapy (HAART), especially on rebound viruses in HIV-1–infected patients.

Among our patients with NAbs against autologous viruses obtained before treatment initiation, we observed virus rebound in 3 patients who had had prolonged suppression of plasma viremia by HAART. To investigate the influence of NAbs on selection of virus quasi species in infected persons during virus rebound after long-term HAART, we analyzed envelope sequences (V1–V5 regions) by comparing preexisting and rebound virus env genotype and assessed their phylogenetic relationships. Envelope (Env) pseudotype viruses generated with plasma HIV-1 env (V1–V4) obtained from these patients were used in examining the difference in neutralizing sensitivity between baseline and rebound viruses. The possible site responsible for neutralizing resistance of rebound viruses was further assessed by using env chimeric viruses. Our observations may aid in understanding the natural evolution of virus quasi species in vivo and may have important implications in the development of new treatment strategies, including antibody-based immunotherapy and an effective vaccine.

Patients and Methods

Patients. Three HIV-infected Japanese patients who had received HAART for a median of 23 months (range, 17–34) and had sustained HIV RNA levels of <50 copies/mL, as measured by the ultrasensitive Amplicor HIV-1 Monitor test (Roche Diag-
Table 1. Profile of human immunodeficiency virus type 1 (HIV-1)–infected patients before highly active antiretroviral therapy (HAART) and at time of virus rebound.

| Patient | HAART regimen | CD4 cells/μL | Before HAART | At time of rebound | HIV-1 RNA copies/mL | Before HAART | At time of rebound | Duration of maximum HIV-1 RNA suppression (<50 copies/mL), months

1 | Sta, Lam, Rtv, Sqv | 252 | 494 | 47,000 | 65,000 | 1
2 | Zid, Lam, Rtv | 172 | 421 | 28,806 | 1000 | 2
3 | Sta, Lam, nFv | 260 | 461 | 760 | 1700 | 3

NOTE. Lam, lamivudine; Nfv, nelfinavir; Rtv, ritonavir; Sqv, saquinavir; Sta, stavudine; Zid, zidovudine.

a Known minimum time during which plasma viremia was ≤50 copies/mL, measured using reverse-transcriptase polymerase chain reaction with a detection limit of 50 copies/mL plasma.

Polymerase chain reaction (PCR) and sequencing. HIV-1 RNA was extracted from 140 μL of plasma by using QIAamp viral RNA mini kits (Qiagen). The envelope gene was reverse transcribed with the Superscript premix amplification system (Gibco BRL Life Technologies) and was amplified by using Taq polymerase (TaKaRa). cDNA was amplified with specific antisense primer M (5′-TAGCCCTTCCAGTCCCCCCTTTTATA-3′) or H (5′-TATGTTCTTCTGCTCTCCAAAGACC-3′) [18]. Nested PCR was done as follows: first-step PCR with primers 1B (5′-AGAAGAGCAGAAGCAGTGCAATGA-3′) and M or H (5′-TATGTTCTTCTGCTCTCCAAAGACC-3′) and second-step PCR with primers 2B (5′-GAGAAGAGCAGAAGCAGTGCAATGA-3′) and F (5′-ATATAATTCCATTCTCAATGGTCTCCCAT-3′) [18]. For both PCR rounds we used GeneAmp PCR System 9600 (Applied Biosystems). PCR products were cloned into the pGEM-T Easy Vector (Promega) by using TA cloning kits (TaKaRa) under conditions specified by the manufacturer.

After being transfected into competent cells, DH5α (TaKaRa), colonies were selected at random and were amplified by using a pair of UCM13 forward and reverse primers (Promega) and then sequenced by using Big Dye Terminator Ready Reaction kits and an ABI 377 automated DNA sequencer (Applied Biosystems). The primers used for sequencing included 2B, 4 (5′-GATACATCCTGCGGATAATCCATGAT-3′), C (5′-TGGCACACAGTGAATGAAATGAAATGGCAATGTA-3′), and F [18]. The sequences were analyzed by Autoassembler sequence analyzer software by comparing the sense and antisense strands of each fragment.

Phylogenetic analysis. Tree reconstructions were done according to a neighbor-joining method implemented with the DNA-DIST program (Kimura distances; transition:transversion ratio, 2.0) and a maximum-likelihood method (version 3.3) implemented with fastDNAml version 1.2.1, applying a transition:transversion ratio of 2.0 and a uniform substitution rate [19, 20]. Pairwise distances from the most recent baseline plasma-derived sequence to rebound virus env sequence were estimated by using the distance matrix program (PHYLIP version 3.573c) [19]. Graphic representation of phylogenetic trees was accomplished with the neighbor-joining/UPGMA method (version 3.572c). The sequence data used in this study were deposited into the DNA Data Bank of Japan under accession numbers AB059279–AB059296 (patient 1), AB059297–AB059316 (patient 2), and AB059317–AB059326 (patient 3).

Generation of recombinant DNA and viruses. Additional recombinant env DNAs (pMOK10, rMOK10, rMOKV1/2, rMOKV3/C3, rMOKV3/C3, rMOKC3, and rMOKC3 for patient 1; pKNI19, pKNI20, pKNI33, rKNI1, and rKNIC3 for patient 2; and pTNA1, pTNA3, rTNA6, rTNA7, rTNA3, and rTNAV2/C3 for patient 3) were generated with representative genotypes of plasma HIV-1 RNA as follows: For pMOK10, rMOK10, pTNA1, pTNA3, rTNA6, and rTNA7, a 0.93-kb Dra I-Bsa BI fragment of plasma HIV-1 env of patients 1 and 3, encompassing the V1–V4 domains, was substituted into the corresponding region of HIV-1 SF162 env in the pSM vector, pSM-SF162 env [21]. For rMOKV1/2, a 0.48-kb Dra III-Bsa BI fragment from the rMOK10 envelope gene and for rMOKV3/C3, a Pvu II-Bsa III fragment (0.22-kb) from the rMOK10 envelope gene were placed in the corresponding regions of pMOK10 env. Chimeric envelopes rMOKV3/C3, rMOKC3, rKNIC3, and rTNAV2/C3 were generated by using a site-directed mutagenesis method according to the procedure recommended by the supplier (Stratagene). The recombinants of pKNI19, pKNI20, pKNI33, and rKNI1 of patient 2 were generated by inducing the Bas BI site without changing the amino acid sequence of the viruses and by letting the Dra III-Bsa BI fragments be placed in the corresponding region of HIV-1 SF162 env, as described above. We confirmed the identity of all chimeras and mutants by DNA sequencing.

The pSM vector–encoding recombinant env of patient plasma HIV-1 (30 μg) was cotransfected with pNL4-3-Lac-E R– (10 μg) [22] and Rev expression vector (2 μg) [21] into 293T cells by the calcium phosphate precipitation method. Reporter plasmid pNL4-3-Lac-E R– was obtained through the National Institutes of Health AIDS Research and Reference Reagent Program. The supernatant, harvested at 24 and 48 h after transfection, was filtered (0.45-μm pore size filters) and was stored in aliquots at −80°C. The concentration of HIV-1 p24 antigen was measured by use of a commercial ELISA kit (ZeptoMetrix). All data points represent the means of quadruplicate determinations, which were then adjusted to identical volumes and used for subsequent neutralization assays.

Cell lines. 293T cells were maintained in Dulbecco’s modified Eagle medium (DMEM; Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS). The CD4+ human osteogenic sarcoma cell line GHOST was maintained in DMEM sup-

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plemented with 10% FBS, G418 (200 μg/mL; Gibco BRL), and hygromycin B (100 μg/mL; Sigma). The GHOST derivatives GHOST-hi5 and GHOST-CXCR4 stably expressed CCR5 and CXCR4, respectively, as described elsewhere [23], and were selected with puromycin (1 μg/mL; Sigma). The HeLa-CD4-LTR-β-gal-CCR5 (MAGI-CCR5) cells [24] were maintained in DMEM supplemented with 200 μg/mL G418, 100 μg/mL hygromycin B, and 100 μg/mL Zeocin (Invitrogen).

**Autologous antibody–mediated neutralization assay.** Antibodies were purified from plasma of the 3 patients and from that of uninfected control persons by using protein A–Sepharose (Bio-Rad). IgG preparations were obtained at 18, 1, and 9 months before viremia rebound from patients 1, 2, and 3, respectively. After MAGI-CCR5 or GHOST-hi5 cells were seeded in 96-well flat-bottom plates (Falcon) at 10^4 cells/well, plates were incubated at 37°C in 5% CO₂ overnight or until they were 70% confluent. We removed the medium and added 5–10 ng of p24 equivalent of chimeric viruses, which corresponded to 500–1000 TCID₅₀, or an IgG/virus mixture in 100 μL to each well. The latter was preincubated for 10–15 min at room temperature. The virus was allowed to absorb for 4 h at 37°C, and 100 μL of the medium, supplemented with Polybrene (20 μg/mL; Sigma) for GHOST-hi5 or DEAE-dextran (10 μg/mL; Pharmacia Biotech) for MAGI-CCR5, was added, without out removal of the inoculum. Infection was noted in 4–6 replicate wells for each experiment by measuring luciferase activity of the cell lysate 48 h after infection. We next removed the culture medium and washed the cells 3 times with PBS (pH 7.4). After thoroughly removing PBS, we added 30 μL of cell-lysing buffer (Luc PGC-50, PicaGene; Wako). We measured luciferase activity with a TR717 microplate luminometer (Applied Biosystems) and substrate (100 μL/well; PicaGene) within 10 s. Percentage of neutralization was calculated by determining the reduction of luciferase activity in the presence of serum antibody, compared with that in control cultures lacking the antibody, which we defined as 0% inhibition. The same assay was done ≥3 times.

**Peptide-binding assay.** V3 (NTI24, NNTIKGHIGPGRAY-AATGSVMGNI) and upstream C3 peptides (SKE28, SKEKWEGLTLQVAIKLAGQFNKSKIDFN) of pMOK10 were synthesized by using an automated peptide synthesizer (Genosys; Sigma). The purity of the preparation was ≥98%. V3, upstream C3, and control peptide (TND27) of HIV-2 strain [25] were coated onto 96-well plates at a concentration of 10 μg/mL and incubated at 4°C overnight. Plates were blocked with 2% bovine serum albumin in PBS for 30 min at room temperature. After a wash with PBS containing 0.1% Tween 20, 100 μL of 1:50 diluted plasma from patient 1, positive control plasma (from an HIV-1–infected LTNP), and seronegative normal human plasma in 2-fold serial dilution (1:50–1:8000) were added and were incubated at room temperature for 2 h. Alkaline phosphatase– conjugated anti-human IgG (ICM Biomedical) diluted 1:1000 in PBS containing 1% FBS was added at 100 μL per well. Assays were developed by addition of substrate. Absorbance was measured at 405 nm. The absorbance was calculated by subtracting the nonspecific reactivity of control wells without peptide.

**Peptide blocking of neutralization.** Neutralizing plasma IgG (200 μg/mL) from patient 1 was preincubated with peptides (NTI24, SKE28, and TND27) at concentrations of 2, 10, and 50 μg/mL for 15 min at room temperature. Aliquots of pMOK10 Env pseudotype virus stocks (5–10 ng of p24) were mixed with IgG mixture that had been preincubated for 10 min at room temperature and inoculated to GHOST hi5 cells. Luciferase activity was measured 48 h after infection, as described above.

**Statistical protocol.** The percentage of neutralization of individual pseudotype viruses was determined for each subject. We used Student’s t test (DA Stats) to compare the mean percentage of neutralization among chimeric viruses. For all statistical tests, significance was 2-tailed with a level of significance of P < .05.

**Results**

**Patients.** We have been monitoring a group of HIV-1–infected patients with NAbs against autologous virus prior to treatment initiation [26]. Virus rebound due to temporal adherence problems was observed in 3 patients after prolonged suppression of plasma viremia by HAART. All 3 are hemophiliacs who were infected by infusion of contaminated blood products ~16 years ago. They were treated with 2 nucleotide reverse transcriptase inhibitors before the initiation of HAART. As shown in table 1, the duration of maximum virus suppression (~50 copies/mL) before viremia rebound was 17, 34, and 18 months for patients 1, 2, and 3, respectively. Numbers of CD4+ T cells increased to >400/μL when the virus rebounded. The level of rebound viremia was higher than that at baseline for patient 1 (6.5 × 10⁴ vs. 4.7 × 10⁴ copies/mL); a low level of virus rebound (referred to as “blips”) was observed for the other 2 patients (patient 2, 10⁴ copies/mL; patient 3, 1.7 × 10⁴ copies/mL). No mutation associated with drug resistance by the pol gene was detected.

**Phylogenetic relationship of rebound viruses with plasma virus quasi species prior to treatment initiation.** The nature of virus rebound after prolonged suppression of plasma viremia has been unclear [27–31], especially for persons who have NAbs against preexisting autologous isolates. Thus, we analyzed the relationship of plasma virus quasi species obtained before initiation of HAART and at the time of virus rebound by sequencing the env gene from the V1–V5 region.

To detect variant frequency changes in such genetically complex populations as the HIV-1 quasi species, plasmid subclones of 10–20 PCR-derived env from plasma of 3 patients were analyzed. In each case, sequences clustered together and were like those of other patients or like prototypic laboratory-adapted and “primary” HIV clones, as analyzed by reconstruction in a maximum-likelihood analysis (data not shown). In each case, the sequences formed individual clusters with highly significant bootstrap values (>80) in the consensus neighbor-joining tree. Figure 1 shows the relationship between baseline and corresponding rebound virus quasi species and pairwise nucleic acid sequence distances in individual phylogenetic trees.

Baseline viruses of patient 2 formed 3 evolutionarily distinct branches: the ancestral cluster (pKN11, 20, and 23), the evolved cluster (pKN140, 43, 8, 35, 16, 24, 21, and 33), and
the intermediate group (pKNI14, 6, 10 and 19; figure 1B). Rebound viruses formed a distinct cluster with a closely related counterpart (pKNI19) in a baseline population with a pairwise distance of 0.05534. The close relationship between the 2 population suggested that rebound viruses in patient 2 possibly had existed in or had evolved from a closely related ancestor of a preexisting population.

For patient 1, the baseline sequences were in 1 subcluster distinguishable from rebound viruses. The distance between the 2 clusters was 1.77619 (figure 1A). The 2 populations in patient 3 gathered in 2 different clusters with a pairwise distance of 3.27075 (figure 1C). The branch lengths from the closest proximal common node of baseline clones (representing the most recent common ancestor) to rebound isolates in patient 3 appeared to be longer than for the other patients. The reemerged viruses in patient 3 may have evolved from a minor variant in the preexisting quasi species.

Comparison of neutralization sensitivity and coreceptor use of baseline and rebound viruses. Because these patients carried the NAb against their autologous HIV-1 isolated before HAART, we attempted to compare the neutralization sensitivity between baseline and rebound viruses against autologous plasma IgG. To construct representative Env pseudotype viruses, we selected clones from each cluster on the basis of the individual phylogenetic tree. Figure 2 shows amino acid sequences of envelope (V1–V5) representatives for each patient.

From patient 1, pMOK10 (11 of 15 baseline clones) and rMOK10 (7 of 20 rebound clones) were selected as representatives (figure 2A). Amino acid differences were observed in V2, V3/C3, V4, and V5 domains. From patient 2, 3 representative clones—pKNI20 (randomly selected from ancestral cluster), pKNI33 (from evolved cluster), and pKNI19 (from the intermediate group)—were selected on the basis of phylogenetic analysis (figure 1B). Of these, we could not establish high-titer infectious virus with pKNI33. Envelope sequences of pKNI20, pKNI19, and rKNI11, selected as representative of the rebound virus (6 of 10 clones), are shown in figure 2B. There were many similarities between rKNI11 and pKNI19 clones, especially in V1/V2 (33 of 35 common aa changes) and V3/C3 domains (15 of 17 common aa changes), as compared with pKNI20. Although a significant difference was also found in the V4 and V5 regions, there were no common changes among these clones. In patient 3, env genotype of both preexisting and rebound viruses showed many similarities within each population. These 2 populations formed different subclusters (figure 1C). We attempted to use predominantly clones of pTNA3 (4 of 9 clones) and rTNA7 (3 of 6 clones) as representatives; however, infectivity of these chimeric viruses was not sufficiently high for neutralization assay. We then used pTNA1 and rTNA6 from each population as representatives and found that there were 2 aa differences between pTNA1 and predominant clone pTNA3 (S39G in V1 and V134A in C2; the first C in the V1 domain was numbered as the first amino acid) and 1 aa difference between rTNA6 and the predominant clone rTNA7 (T24I in V1). Most differences in envelope sequence were located in the V1 region between pTNA1 and rTNA6 (14 of 20 aa changes; figure 2C) and between pTNA3 and rTNA7 (12 of 17 aa changes).

**Figure 1.** Phylogenetic trees for envelope sequences obtained from plasma virion RNA at baseline and in rebound viruses of patients 1–3 (A–C, respectively). Phylogenetic relationships among the V1–V5 regions of envelope sequences were estimated by neighbor-joining methods (distance matrix program, version 3.573c [19]). Envelope sequences of pMOK10, pKNI11, and pTNA3 were used to root the tree. Dotted boxed regions show cluster of rebound viruses. Stability of nodes was assessed with the bootstrap value (>80% in all nodes). Horizontal branch lengths reflect genetic distance between sequences. Nos. in parentheses represent pairwise distance between adjacent nodes.
To examine the differences between baseline and rebound viruses in neutralization sensitivity against autologous antibodies, Env pseudotype viruses, constructed with each representative clone env, as described in Patients and Methods, were used in neutralization assays with target MAGI-CCR5 or GHOST-hi5 cells. Both baseline (pMOK10) and rebound viruses (rMOK10) were sensitive to autologous antibody–mediated neutralization, as compared with the nonspecific inhibition exerted by seronegative control IgG (figure 3A). However, the sensitivity was significantly higher for pMOK10 than for rMOK10 at every IgG concentration (P = .025, .045, and .0028 at IgG concentrations of 100, 200, and 400 µg/mL, respectively). As shown in figure 3B, significant neutralization by autologous IgG was observed for baseline virus pKNI20 from patient 2. In contrast, no significant inhibition was detected for the rebound virus rKNI1 at 100 and 200 µg/mL IgG and its closely related counterpart, pKNI19 (P = .001 and .01, IgG concentrations of 100 and 500 µg/mL), compared with pKNI20. A representative virus from baseline populations for patient 3 (pTNA1) was sensitive to autologous IgG–mediated neutralization, and the rebound virus rTNA6 appeared to be resistant to neutralization at IgG concentrations of 100 and 200 µg/mL (P = .02; figure 3C). These results suggest that the rebound viruses had become resistant to the autologous IgG–mediated neutralization, compared with baseline viruses, in these patients. The rebound viruses may have emerged from the population that escaped neutralization, which possibly existed in or evolved from a minor variant in baseline quasi species.

Understanding coreceptor use of HIV-1 during potent antiretroviral therapy may have a role in virologic monitoring of patients, especially for neutralization-resistant viruses that emerge.
We examined coreceptor utilization of baseline and rebound viruses by comparing the infectivity of each pair of Env pseudotype viruses (by patient) to GHOST-hi5, GHOST-CXCR4, and GHOST parental cells, by use of an infection assay. Both baseline and rebound viruses used CCR5 as a coreceptor exclusively in all 3 patients (data not shown). The data indicate that changes in neutralization sensitivity were not accompanied by a shift in coreceptor use.

### Association of amino acid changes in the V3/C3 and/or V1/V2 region of gp120 with resistance of rebound virus against autologous IgG

To identify the domain responsible for conferring the relative resistance to the antibody-mediated neutralization, chimeric viruses with the V1/V2 (rMOKV1/2) or V3/C3 regions (rMOKV3/C3) of rMOK10 were constructed with the backbone envelope sequence of pMOK10 for patient 1 (figure 4A). Neutralization sensitivity was compared by in-
cubating the chimeric viruses with autologous IgG at a concentration of 200 μg/mL before infecting the target MAGI-CCR5 cells. Percentage of neutralization was calculated as described in Patients and Methods. As shown in figure 4B, neutralization resistance of rMOK10 was detected when compared with pMOK10 (P < .001). A comparable level of neutralization resistance was observed with rMOKV3/C3 (P < .002) but not with rMOKV1/2 (P = .22).

To further determine the site in rMOKV3/C3 responsible for the neutralization resistance, additional mutant viruses that contained the V3 sequence (rMOKV3) or the upstream C3 sequence (rMOKC3) of rMOK10 were constructed by site-directed mutagenesis (figure 4A). A relative resistance was observed for both rMOKV3 and rMOKC3, as compared with pMOK10 (P = .0002 and < .0001, respectively; figure 4B). Repeated experiments showed that neutralization resistance induced by rMOKC3 was nearly at the same level as that induced by rMOK10, but rMOKV3 showed a lesser degree of resistance than seen with rMOKC3.

For patient 2, mutant chimeric viruses rKNIV1/2 and rKNIV3/4, containing the V1/V2 or the V3/V4 region of rKN1 in the backbone of pKNI20 env, were constructed. However, high-titer infectious viruses could not be obtained. We then prepared mutant pseudotype virus rKNIC3, which contained rKN1 upstream from C3 (amino acids were changed from pKNI20 S239 to rKN1 N239, R240G, N242E, and T244N), with the backbone envelope sequence of pKNI20, using site-directed mutagenesis.

As shown in figure 5A, partial but significant resistance against autologous IgG neutralization was observed with rKNIC3 virus (P = .011). Similarly, we prepared rTNA6 and rTNAV2/C3, which contained rTNA6 upstream from C3 (T252N) or both rTNA6 upstream from C3 and V2 (D179N and S182R), respectively, with the backbone envelope sequence of pTNA1 from patient 3. Since the rTNAC3 mutant virus did not have sufficient infectivity for our assay, we used the rTNAV2/C3 mutant virus to examine neutralization sensitivity. As shown in figure 5B, the rTNAV2/C3 mutant remained sensitive to autologous IgG. Because amino acid differences between rTNA6 and rTNAV2/C3 were mostly located in the V1 region (14 in the V1 region and 3 in the C2 domain; figure 2), one can reasonably assume that amino acid changes in the V1 region were responsible for the neutralization resistance of rTNA6. These results indicate that amino acid changes in the upstream C3 region contributed principally to neutralization resistance against autologous IgG in patient 1 and partially in patient 2. However, changes in the V1 region likely conferred neutralization resistance to rebound viruses in patient 3.

Involvement of antibodies against V3 epitope(s) in neutralization of pMOK10. Because neutralization resistance was associated with amino acid changes in the upstream C3 region in patient 1, we investigated whether antibodies against upstream C3 and/or V3 epitopes were involved in the neutralization of pMOK10. Synthetic peptides corresponding to the V3 region (NTI24) and to the upstream C3 domain (SKE28) of pMOK10 and to a control peptide (TND27) were subjected to solid-phase ELISA to detect binding activities of plasma antibodies to V3 or upstream C3 epitopes. As shown in figure 6A, reactivity of plasma antibodies from patient 1 was not evident (only marginal reactivity was observed at the highest concentration) to either V3 or upstream C3 peptides.

We then tested the inhibitory activity of the peptides against the neutralizing activity of patient 1 plasma IgG with pMOK10 chimeric virus. Peptides at concentrations of 2, 10, and 50 μg/mL were preincubated with autologous IgG (300 μg/mL) before reaction with the pseudotype virus. As shown in figure 6B, partial neutralization inhibition was detected in the presence of a higher concentration of the V3 peptide (P = .18 and .04 for 10 and 50 μg/mL, respectively, vs. the control peptide at the same concentration), but no significant differences were observed with the upstream C3 and control peptides. The apparent discrepancy between the reactivity and neutralization inhibition may partly be due to the assay condition, in which the V3 peptide might reconstitute native conformation in liquid-phase assay but not in solid-phase ELISA. Thus, antibodies against V3 epitope(s) may have contributed to antibody-mediated neutralization in patient 1.
Discussion

The emergence of neutralization-resistant HIV variants was noted in in vitro experiments and in an animal model system that used monoclonal or polyclonal antibodies [6, 9, 11, 32]. However, the polyclonal nature of the virus quasi species and NAbs made further analysis difficult for HIV-infected persons. We had a unique opportunity to characterize rebounding viruses in the presence of autologous NAbs after prolonged suppression by HAART. In this study, rebound viruses from all 3 patients became resistant to neutralization by autologous IgG, unlike the baseline viruses. This observation is consistent with earlier reports, which described a phenomenon of relative resistance of later isolates to autologous antibody neutralization, in contrast to earlier isolates [4, 5] obtained before HAART was administered to patients.

Comparison of the corresponding sequences of baseline and rebound viruses revealed domains related to selection by patient NAbs in vivo. For patient 1, although widespread changes in amino acids between pMOK10 and rMOK10 were observed, the responsible domain was identified in the V3/C3 (upstream) region when we used the Env pseudotyping assay with a chimeric envelope. Further analysis with rMOKV3 and rMOKC3 env recombinant viruses showed that the principal involvement was in the upstream C3 domain (figure 4B). Sequence comparison between neutralization-sensitive clone pKNI20 and 2 resistant clones (pKNI19 and rKNI1) from patient 2 showed that the major amino acid difference was in the V1/V2 and V3/C3 (upstream) regions. However, mutant pseudotype virus rKNIC3, which had the upstream C3 region genotype of rKNI1 in the backbone of pKNI20 virus env, showed partial neutralization resistance against autologous IgG (figure 5A). Net charge increases were also observed in the upstream C3 region of these neutralization-resistant clones in both patients (table 2). The net charge increase in this upstream C3 domain in neutralization-resistant viruses, to our knowledge, has not been previously described. Although the position of amino acid substitutions in our patients differed from that described in previous reports, the importance of changes in the C3 domain when determining neutralization sensitivity to the MAb against the CD4 binding site of gp120 or polyclonal human serum in vitro has been demonstrated [6, 9, 32, 33].

Net charge changes in the C3 region may possibly alter the epitopes recognized by neutralization antibodies in a conformational manner. Neutralization escape from polyclonal sera occurs through alterations at several different epitopes resulting from single amino acid substitution in the C3 domain, which influence envelope conformation [6, 9]. These observations suggest the importance of amino acid changes in upstream C3 in HIV-1 neutralization sensitivity to autologous antibodies.

Amino acid changes between neutralizing-sensitive clone pTNA1 and resistance clone rTNA6 for patient 3 were observed mostly in the V1/V2 region, with a 1-site decrease in N-linked glycosylation site. Further analysis with mutant virus rTNAV2/C3 suggested that mutation in the V1 region like-
ly contributed to neutralization resistance in this patient. This observation is consistent with reports that mutation in the V1/V2 region is associated with resistance to some MAbs, including those that recognize some conformational epitopes [32–35].

For patient 1, the antibody-mediated neutralization was also partially inhibited by the V3 peptide (figure 6B), but both V3 and C3 peptides were poorly recognized in solid-phase ELISA (figure 6A). Although these results do not exclude the role of linear epitopes in neutralization, it may be that the V3 peptide was recognized by the antibody in liquid phase as part of the conformational epitope, and its conformational nature resulted in a poor binding reaction with IgG in solid phase. As reported elsewhere, the C3 peptide did not form a linear epitope but participated in the conformational epitope(s) [32, 33]. The upstream C3 domain contributed primarily to the conformational epitope(s) recognized by autologous antibodies. Changes in this region significantly facilitated emergence of the neutralization escape mutants in HIV-1–infected subjects. The V3 domain also might have partially contributed to the neutralization resistance by participating in the conformational epitope(s) recognized by MAbs. Therefore, changes in upstream C3 in neutralization resistance of rebound HIV-1 to the autologous antibody are important.

The origin of the rebound viruses has been investigated by comparing the genotype of the rebounding virus with that in the latent reservoir [27–29]. In agreement with a previous study [30], we observed a close relationship between rebound viruses and baseline quasi species. In patient 2, the genetic distance between the 2 populations was short enough to assume that rebound viruses or their closely related variants had existed in baseline quasi species prior to the initiation of HAART. However, phylogenetic analyses also suggested the evolution of rebound viruses from a minor variant of baseline quasi species for patients 1 and 3. However, one would need to exclude the possibility that all of the variants had existed in peripheral blood or other lymphoid organs either latently or with low-level replication in the infected persons. We detected only a small population of plasma HIV-1, so it was difficult to draw any firm conclusion based on the limited genetic findings. Our data suggest the possibility that rebound viruses resulted from a selection of preexisting quasi species by MAbs. Thus, various host factors, including autologous MAbs, should be taken into account when evaluating the phylogenetic relationship among HIV-1 populations.

Our data provide further evidence about immunologic selection of virus quasi species by autologous MAbs in the course of infection, even under conditions of prolonged virus suppression by HAART. On the basis of our results, along with other published data, it is reasonable to assume that the antibody-mediated selection of virus quasi species is associated with some conformational epitopes, including the V3/C3 and/or V1/V2 regions of the envelope in HIV-1–infected persons. Further analysis of anti-HIV immune responses and the natural evolution of virus quasi species in infected persons may have important implications in the development of vaccines and immunotherapy.

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Table 2. Summary of glycosylation sites and net charges.

<table>
<thead>
<tr>
<th>Chimeric clone</th>
<th>No. of glycosylation sites</th>
<th>V1/V2 domainsa</th>
<th>V3 loopb</th>
<th>Upstream C3c</th>
<th>V1/V2 domainsa</th>
<th>V3 loopb</th>
<th>Upstream C3c</th>
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</table>

NOTE. Nos. are amino acid positions according to GenBank HXB2 sequence.

a Regions of amino acid sequences of env gp120 related to neutralizing resistance: 126C–196C.

b Regions of amino acid sequences of env gp120 related to neutralizing resistance: 296C–331C.

c Regions of amino acid sequences of env gp120 related to neutralizing resistance: 331C–364S.

References


ERRATA

In an article in the 15 January 2002 issue of the Journal (Lundgren JD, Mocroft A, Gatell JM, et al. A clinically prognostic scoring system for patients receiving highly active antiretroviral therapy: results from the EuroSIDA study. J Infect Dis 2002;185:178–87), there is an error in the sixth paragraph of the Results section. The virus loads listed in the second sentence should be as follows: <500, 500–9999, and ≥10,000 HIV RNA copies/mL [not <500, 500–999, and ≥1000 HIV RNA copies/mL]. The Journal regrets this error.

In an article in the 1 March 2002 issue of the Journal (Wang F-X, Kimura T, Nishihara K, Yoshimura K, Koito A, Matsushita S. Emergence of autologous neutralization-resistant variants from preexisting human immunodeficiency virus (HIV) quasi species during virus rebound in HIV type 1–infected patients undergoing highly active antiretroviral therapy. J Infect Dis 2002;185:608–17), there are errors in table 1. In the column labeled “Duration of maximum HIV-1 RNA suppression,” the data for patients 1, 2, and 3 should be 17, 34, and 18 months, respectively [not 1, 2, and 3 months]. The Journal regrets this error.