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Viremia Control Despite Escape from a Rapid and Potent Autologous Neutralizing Antibody Response After Therapy Cessation in an HIV-1-Infected Individual[1]

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The neutralizing Ab response after primary HIV-1 infection is delayed relative to the virus-specific CD8$^+$ T cell response and the initial decline in plasma viremia. Because nearly all HIV-1 infections result in AIDS, it would be instructive to study cases where neutralizing Ab production commenced sooner. This was done in subject AC10, an individual treated during early infection and in whom a rapid autologous neutralizing Ab response was detected after therapy cessation as rebound viremia declined and remained below 1000 RNA copies/ml of blood for over 2.5 years. This subject's Abs were capable of reducing the infectivity of his rebound virus by >4 logs in vitro at a time when rebound viremia was down-regulated and virus-specific CD8$^+$ T cells were minimal, suggesting that neutralizing Abs played an important role in the early control of viremia. The rebound virus did not exhibit an unusual phenotype that might explain its high sensitivity to neutralization by autologous sera. Neutralization escape occurred within 75 days and was proceeded by neutralizing Ab production to the escape variant and subsequent escape. Notably, escape was not associated with a significant rise in plasma viremia, perhaps due to increasing CD8$^+$ T cell responses. Sequence analysis of gp160 revealed a growing number of mutations over time, suggesting ongoing viral evolution in the face of potent antiviral immune responses. We postulate that an early effective neutralizing Ab response can provide long-term clinical benefits despite neutralization escape. ***The Journal of Immunology*****, 2003, 170: 3906–3914.**

Primary infection with HIV-1 is accompanied by a peak in plasma viremia that soon declines as the antiviral immune response develops (1, 2). Setpoint levels of viremia are predictive of clinical outcome (3) and the likelihood of subsequent virus transmission (4, 5). The types of immune responses associated with early viremia control may be valuable to augment for therapeutic immunization and critical to generate by vaccines that aim to control rather than prevent infection. Much emphasis has been placed on the importance of virus-specific CD8$^+$ T cells, which are present during primary infection (6–9) and show a strong association with long-term stable clinical status (10, 11) and at least temporary viremia control after treatment cessation in subjects who initiate highly active antiretroviral therapy (HAART)[3] early in infection (12, 13). Direct evidence for their protective role in vivo was obtained recently when SIV-infected macaques exhibited dramatic rises in viremia after experimental CD8$^+$ T cell depletion (14, 15). These combined data provide a strong rationale for HIV-1 vaccines that elicit CD8$^+$ T cell responses (16). Increasing evidence in the simian-human immunodeficiency virus (SHIV) model suggest that, while such vaccines are not able to prevent infection altogether, they are nonetheless partially effective at suppressing viremia and attenuating disease (17–20). Notably, similar vaccine strategies have had a weaker impact on less neutralizable SIV strains (21–23).

It might be necessary to improve the efficacy of current HIV-1 vaccine candidates by incorporating an effective neutralizing Ab component. The importance of neutralizing Abs was made clear when their passive administration at the time of virus exposure or shortly thereafter prevented SIV and SHIV infection in macaques (24–29). Complete protection from infection is a highly desirable goal for HIV-1, which integrates genetically and is difficult to eliminate from the body. However, neutralizing Abs induced by candidate vaccines wane rapidly and may only present at sufficient levels to prevent infection for a short period of time. As an alternative strategy, appropriate B cell priming might hasten neutralizing Ab production and add to the benefit of virus-specific CD8$^+$ T cells after infection. There have been very few cases identified in which to investigate the possible virologic and clinical benefits of a rapid neutralizing Ab response in HIV-1 infection, as neutralizing Ab production is delayed and does not coincide with the decline in viremia during primary infection in the vast majority of cases (30–34).

We recently reported a case of rapid autologous neutralizing Ab production after treatment cessation in an HIV-1-infected individual (AC10) in whom HAART was initiated early in infection and who discontinued treatment after 1.5 years (35). Those neutralizing Abs correlated with a dramatic decline in rebound viremia and the maintenance of low virus loads for at least 8 mo. The kinetics and magnitude of neutralizing Ab production in this subject were consistent with a secondary response that resulted from earlier B

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[3] Abbreviations used in this paper: HAART, highly active antiretroviral therapy; SHIV, simian-human immunodeficiency virus; STI, structured treatment interruption; $TCID_{50}$, tissue culture infectious dose 50; SFC, spot forming cell; TCLA, T cell line-adapted; sCD4, soluble CD4.

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cell priming. We hypothesize that priming took place before viremia was controlled by HAART and was preserved and possibly augmented while on HAART. This subject has now remained off therapy for over 2.5 years. The virologic and immunologic profile of this subject was investigated in greater detail to gain a better understanding of the potential value of neutralizing Abs after the cessation of early HAART.

Materials and Methods

Study subjects

Subject AC10 is a male Caucasian, 35 years of age, who was diagnosed with HIV-1 in March of 1998. Transmission was believed to be through sex with another male. This subject was weakly seropositive by ELISA and Western blot and had 40,700 copies of viral RNA/ml of blood at the time of diagnosis (13, 35). HAART was initiated at this time and was discontinued 1.5 years later. Plasma viremia was below the limit of detection for 16 mo before treatment cessation. Subjects JAF-01, NLS-02, LEH-03, SCE-06, and PRH-07 were part of a structured treatment interruption (STI) study aimed at augmenting their natural antiviral immunity (36). These latter subjects initiated HAART during chronic infection and underwent two STIs of 1-mo duration separated by 1 mo of HAART before a final 3-mo STI.

Viruses, serum samples, and coreceptor phenotyping

A virus isolate obtained during the peak of rebound viremia from subject AC10 was described previously (35). This virus was isolated 44 days after treatment cessation and possesses an R5 phenotype. We designate this isolate AC10.44 to reflect the time of isolation. Additional isolates from AC10 were obtained by PBMC coculture as described (37). Virus-containing culture fluids were collected at the peak of p24 Gag Ag production and made cell-free by 0.45-micron filtration. Cell-free virus was stored in 1-ml aliquots at −80°C. All viruses were used as either original isolation fluids or as a single passage in PBMC. Infectious titers were measured in tissue culture infectious dose 50 ($TCID_{50}$) assays in PBMC calculated as described (38). Biologic phenotypes were assigned according to established nomenclature (39) and determined by differential infection in U87-CD4-CCR5, U87-CD4-CXCR4, and MT-2 cells (40).

Cloning and sequencing

Genomic DNA was extracted from infected PBMC (infected in vitro) by using a Blood and Cell Culture DNA kit (Qiagen, Valencia, CA). Complete HIV-1 *env* genes were amplified using 0.1-μg genomic DNA with the primers *env*1Atopo A 5′-CACCGGCTTAGGCATCTCCTATGGCAG GAAGAA and env1N 5′-TAGCCCTTCCAGTCCCCCCTTTTCTTTTA as previously described (41). Thermocycling for PCR was as follows: one cycle at 94°C for 1 min; 30 cycles of a denaturing step at 94°C for 30 s, an annealing step at 55°C for 30 s, an extension step at 68°C for 5 min and, lastly, one cycle of an additional extension at 68°C for 7 min. PCR products were visualized on a 0.7% agarose gel and purified with a QIAquick Gel Extraction kit (Qiagen). Purified PCR products were cloned directly into a pcDNA3.1/V5-His-TOPO vector (Invitrogen, Carlsbad, CA). The ligation reaction was used to transform INVαF′ cells. Plasmid DNA was extracted from transformed cells using the Qiagen plasmid kit and sequenced on an automatic sequencer ABI 3100 (Applied Biosystems, Foster City, CA) by using the cycle-sequencing and dye terminator methods. To avoid a sequencing error, both DNA strands for two or three independent clones of each Env were sequenced using a primer-walking method. The raw sequence data were edited using Sequencher program (version 4.2; Gene Codes, Ann Arbor, MI).

ELISA

V3-specific binding Abs were assessed in Nunc MaxiSorb F96 Immuno plates (Roskilde, Denmark) coated with peptide at 0.5 μg/ml as described previously (42). An AC10.44 V3 peptide (RPNNNTRKGIHIGPGRAFYT TGDII) was synthesized and purified by SynPep (Dublin, CA). The peptide was judged >90% pure by high pressure liquid chromatography and mass spectrometry. ELISAs were performed in duplicate with a 1/100 dilution of test samples.

Neutralizing Ab assays

Neutralization assays with primary HIV-1 isolates were performed with a fixed dose of virus in PBMC as described (35). Briefly, test samples were serially diluted 3-fold in triplicate in 96-well U-bottom culture plates. Cell-free virus was added (500 $TCID_{50}$/well) and incubated for 1 h at 37°C. Mitogen-stimulated PBMC in IL-2-containing growth medium (RPMI 1640 containing 20% heat-inactivated FBS, 50 μg gentamicin/ml, and 5% human IL-2) were added at 5×10^5 cells/well and incubated overnight. Cells were washed four times and suspended in 150 μl of growth medium. Viral p24 in culture supernatants was quantified by Ag capture ELISA (PerkinElmer Life Sciences, Boston, MA) at a time when virus production in control wells (no test sample) was in an early linear phase of increase (usually 3–4 days), which is when optimal sensitivity is achieved (43). Titers of neutralizing Abs are reported as the highest serum dilution to reduce p24 synthesis by 80% relative to the amount of p24 synthesized in control wells. All serum samples were heat-inactivated for 1 h at 56°C before assay.

$TCID_{50}$ reduction assays were performed by incubating cell-free virus (>5×10^5 $TCID_{50}$/ml) with various dilutions of serum samples for 1 h at 37°C. Following incubation, the virus/serum mixtures were diluted 25-fold in growth medium and further diluted serially 5-fold in quadruplicate in 96-well U-bottom plates. PBMC in IL-2-containing growth medium were added (5×10^5 cells/well) and incubated overnight. Cells were washed four times and suspended in 150 μl of fresh IL-2-containing growth medium. Viral p24 Ag synthesis was monitored by ELISA 10 days later. Wells containing >0.1 ng p24/ml were considered positive for the calculation of $TCID_{50}$ (38).

Neutralization of HIV-1_{IIIB} and HIV-1_{MN} was measured in MT-2 cells as described (44). Neutralization titers in the MT-2 assay are the serum dilution at which 50% of cells were protected from virus-induced killing as measured by neutral red uptake. A 50% protection from cell-killing corresponds to an ~90% reduction in p24 synthesis in this assay (45). For V3 peptide blocking experiments, serum samples were diluted with an equal volume of sterile PBS, pH 7.4, and incubated for 1 h at 37°C in the presence and absence of V3 peptide (50 μg/ml) before performing neutralization assays as described above.

Characterization of HIV-1-specific T cell responses

HIV-1-specific $CD8^+$ T cell responses in study subject AC10 were quantified by ELISPOT assay as previously described (46), using a panel of 410 overlapping peptides (16–19 mer peptides overlapping by 10 aa) spanning the entire expressed HIV-1 clade B consensus sequence, as well as 28 peptides corresponding to optimal CTL epitopes described for the study subject's HLA class I type (A2/24, B38/60, Cw2, 12) (47). In brief, PBMC were plated at 100,000 cells per well with peptides at a final concentration of 10^{-5} M in 96-well polyvinylidene difluoride-backed plates (MAIP S45; Millipore, Bedford, MA) precoated with 0.5 μg/ml anti-IFN-γ mAb, 1-DIK (Mabtech, Stockholm, Sweden) overnight at 4°C. For negative controls 100,000 cells were incubated with R10 alone, for positive controls with PHA. The plates were incubated overnight (14–16 h) at 37°C, 5% CO_2, washed, and then biotinylated anti-IFN-γ mAb 7-B6-1 (Mabtech) was added at 0.5 μg/ml and incubated at room temperature for 90 min. Following washing, 100 μl of 1/2,000-diluted streptavidin-conjugated alkaline phosphatase (Mabtech) was added per well at room temperature for 45 min. Individual IFN-γ-producing cells were detected as dark spots after a 20–30 min color reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Bio-Rad, Hercules, CA). The number of specific IFN-γ-secreting T cells was counted by direct visualization and calculated by subtracting the negative control value and expressed as spot forming cells (SFC) per 10^6 input cells. Negative controls were always <30 SFC per 10^6 input cells. Responses three times above mean background activity and >50 SFC/10^6 PBMC were considered positive. $CD8^+$ T cell dependence of responses was determined by CD8 depletion studies.

Results

Serologic, virologic, and clinical profile

Subject AC10 exhibited relatively weak seroconversion by Western blot before treatment (week −73) that did not increase during therapy but intensified dramatically after treatment cessation (Fig. 1). This profile is consistent with early seroconversion at the time of enrollment. Plasma viremia was below detection during therapy and rebounded to a peak of ~5000 RNA copies/ml on days 40 and 44 after treatment cessation (35). Viremia declined to undetectable levels 1 wk later but became detectable again by day 65 and remained detectable at very low levels (<1000 RNA copies/ml) at all time points examined for 2.6 years (Fig. 2). Average virus loads after the decline in rebound viremia were 333 RNA copies/ml. Subject AC10 remains healthy with $CD4^+$ T cell counts >600/μl at the time of this writing.

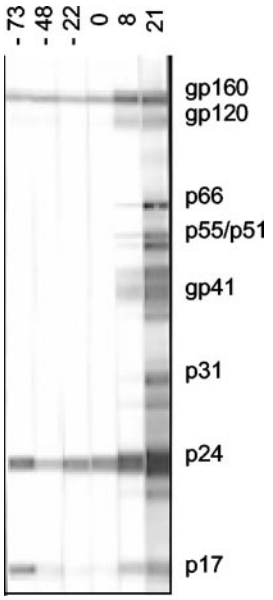

FIGURE 1. HIV-1 Western blots. Serum obtained before treatment and at multiple time points before and after treatment cessation were assessed by Western blot (DuPont). Values above each strip indicate the number of weeks before treatment cessation. Treatment was initiated immediately after the wk −73 serum collection. Treatment was stopped at week 0.

Serum obtained as early as 58 days after treatment cessation was previously shown to contain high titers of neutralizing Abs measured against the rebound virus isolate; those neutralizing Abs were shown to be relatively strain-specific, having no activity against T cell line-adapted (TCLA) strains and rarely neutralizing heterologous primary isolates (35). Serum samples collected 734, 923, and 970 days after therapy cessation maintained an ability to neutralize the rebound isolate (Fig. 2). Despite this sustained response and opportunities for affinity maturation, the 923-day serum sample neutralized only one of eight heterologous clade B primary isolates tested (data not shown).

Serum samples and the rebound isolate from subject AC10 were studied in greater detail to gain a better understanding of the nature and possible benefits of a rapid autologous neutralizing Ab response in HIV-1 infection. The high titers of neutralizing Abs in this subject suggested a potent response but it was not clear how

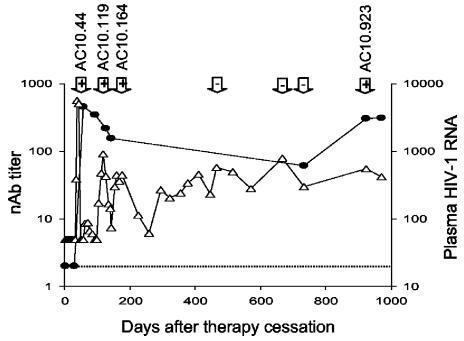

FIGURE 2. Profile of plasma viremia and autologous neutralizing Ab production in subject AC10. HAART was initiated early in infection and was discontinued 1.5 years later. Neutralizing Abs were measured in a fixed virus dose assay in PBMC using serum samples that were obtained after therapy cessation. The virus in this case was isolated 44 days after treatment cessation (AC10.44). Arrows indicate time points and outcomes of all virus isolation attempts. The dashed line represents the limit of detection in the neutralization assay.

Table I. *Autologous neutralization potency measured with a fixed virus dose compared to $TCID_{50}$ reduction*

Virus[a]	Autologous Serum[b] (days)	Serum Dilution for $TCID_{50}$ Reduction Assay	$TCID_{50}$ Reduction[c]
AC10.44	58	1/5	>10,000-fold
		1/25	456-fold
		1/125	14-fold
	734	1/5	>10,000-fold
	923	1/5	>10,000-fold

[a] Virus AC10.44 was isolated during the peak of rebound viremia, 44 days after treatment cessation.
[b] Autologous serum samples from subject AC10 were obtained at the indicated days after treatment cessation.
[c] $TCID_{50}$ reduction assays were performed with the indicated dilution of serum samples.

much virus could be neutralized. For example, titers of neutralizing Abs measured in the fixed virus dose assay are based on a 5-fold reduction in infectivity of a 500 $TCID_{50}$ inoculum, which is the minimum reduction to yield a true positive outcome in our assay (45). As another measure of neutralization potency, high concentrations of virus (>10^6 $TCID_{50}$) were incubated with test serum, after which time the surviving fraction of infectious virus particles was quantified in $TCID_{50}$ assays. Serum obtained on days 58, 734, and 923 after treatment cessation were evaluated in this way with rebound isolate AC10.44. A 1/5 dilution of all serum samples reduced infectivity by >4 logs (Table I). Moreover, a reduction of >2 logs was seen at a 1/25 dilution and a reduction of at least 1 log was seen at a 1/125 dilution of the 58-day serum sample (Table I).

We next assessed the general neutralization sensitivity of isolate AC10.44 to see whether it exhibited a TCLA-like phenotype that might explain its potent neutralization by autologous sera. TCLA strains are highly sensitive to neutralization relative to primary isolates with a variety of reagents that include soluble CD4 (sCD4) and heterologous HIV-1-positive serum samples (45, 48). TCLA strains also exhibit a greater sensitivity to neutralization by mAbs such as IgG1b12, 2G12, and 2F5 in most but not all cases (49–53). As shown in Table II, isolate AC10.44 resisted neutralization by high concentrations of sCD4 and all three mAbs Abs (ID_{80} >50 μg/ml). Moreover, the isolate was only moderately sensitive to HIV-1-positive serum samples that contained high titers of neutralizing Abs to TCLA strains (45); these sera also were capable of neutralizing the autologous isolate from the respective donor (36) and neutralize heterologous primary isolates sporadically and with

Table II. *Neutralization of rebound isolate AC10.44 with standard reagents*

Reagent[a]	ID_{80}[b]
sCD4	>50 μg/ml
IgG1b12	>50 μg/ml
2G12	>50 μg/ml
2F5	>50 μg/ml
HIV-1^+ sera	
JAF-01	1/6
NLS-02	1/112
LEH-03	1/36
SCE-06	<1/5
PRH-07	1/5

[a] HIV-1^+ plasma samples were obtained during a period of treatment interruption from chronically infected individuals.
[b] Neutralization assays were performed with a fixed dose in PBMC. ID_{80} is either the lowest concentration of sCD4, IgG1b12, 2G12, and 2F5 or the highest dilution of serum to reduce p24 synthesis by 80%.

low potency (45). These results demonstrate that rebound virus from subject AC10 is not unusually sensitive to neutralization by a variety of standard indicator reagents.

V3-specific neutralizing Abs

The V3 loop of gp120 was examined as a potential target for neutralizing Abs in serum samples from subject AC10. V3 is a major target for the neutralization of TCLA strains (54, 55) but is rarely a target for primary isolate neutralization (45, 56–58). Serum samples were first examined by ELISA for the presence of V3-specific binding Abs. Serum obtained 58 days after treatment cessation that possessed potent autologous neutralizing activity reacted poorly to the AC10.44 V3 peptide (Fig. 3), suggesting that most neutralizing activity against the rebound virus was non-V3-specific. ELISA reactivity was much stronger with AC10 serum obtained 734 days after treatment cessation, resembling the reactivity of serum samples from chronically infected individuals (Fig. 3).

To determine whether the V3-specific Abs detected in AC10 serum possessed neutralizing activity, the 58- and 734-day serum samples were preincubated with an AC10.44 V3 peptide (50 μg/ml) before performing neutralization assays. As shown in Fig. 4, the peptide failed to block neutralization of rebound isolate AC10.44 in both cases but blocked all detectable neutralization of HIV-1_{MN} and a major portion of neutralization of HIV-1_{IIIB} by the 734-day serum sample. The 58-day serum sample was not tested because it did not neutralize TCLA strains. These results demonstrate that V3-specific Abs in late serum from subject AC10 were capable of neutralizing TCLA strains but not the autologous virus. The V3 loop of isolate AC10.44 appears to exhibit a phenotype typical of most primary isolates in this regard. Poor exposure of epitopes in the V3 loop of primary isolates compared with TCLA strains is common and may result from either the addition of *N*-linked glycans or the structure of tertiary folds on the gp120 molecule (45, 56–59). We do not wish to imply that all V3-specific neutralization epitopes on isolate AC10.44 were masked. For example, a portion of neutralizing Abs might have bound epitopes that possess a higher order structure, in which case linear peptides would not compete with the corresponding conformation-dependent Abs.

CD4 dependency

A small number of HIV-1 strains have been identified that do not require CD4 for cell entry, and all of those strains exhibit an unusually

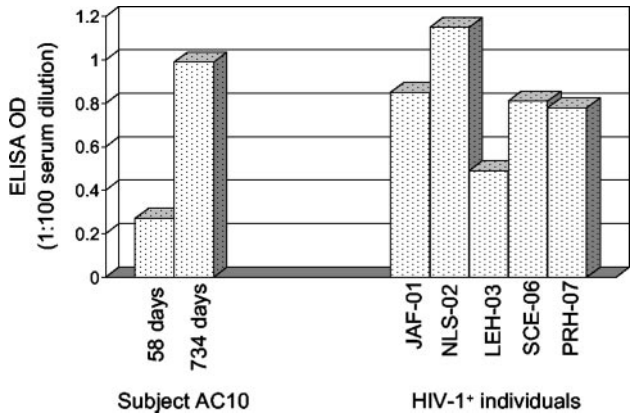

FIGURE 3. V3-specific binding Abs. Serum samples from subject AC10 collected at 58 (□) and 734 days (■) after treatment cessation were assessed by ELISA for V3-specific binding Abs. Additional serum samples came from healthy HIV-1-infected individuals who participated in STI as described in the text. Serum samples from these latter subjects were obtained after the final STI. All serum samples were assayed at a 1/100 dilution in duplicate.

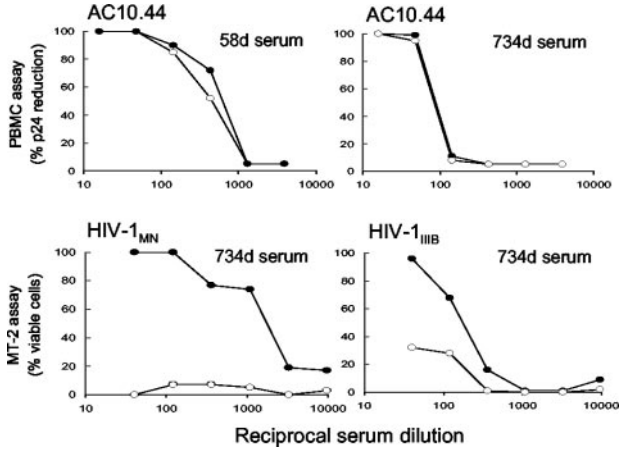

FIGURE 4. Detection of V3-specific neutralizing Abs. V3-specific neutralizing Abs were sought by assaying serum samples in the presence and absence of a V3 peptide (50 μg/ml) corresponding to rebound isolate AC10.44. Serum samples were collected 58 and 734 days after treatment cessation from subject AC10. Neutralization of isolate AC10.44 was measured with a fixed virus dose in PBMC. Neutralization of TCLA strains, HIV-1_{MN} and HIV-1_{IIIB}, was measured in MT-2 cells. ●, Without peptide; ○, with peptide.

high degree of sensitivity to neutralization (60–63). Therefore, it was of interest to determine the CD4 dependency of rebound isolate AC10.2. This was done by testing infection in BC7-CCR5 cells. BC7-CCR5 cells lack CD4 but express CCR5 and are permissive to infection by CD4-independent strains of SIV (64) and HIV-1 (C. LaBranche, unpublished observation). As shown in Table III, no infection of BC7-CCR5 cells was detected with isolate AC10.44 for up to 12 days of incubation, whereas high-level infection was detected within 6 days in PBMC. Parallel infection assays with the CD4-independent R5 strain NL-ADA (65) produced high levels of infection in both cell types, thereby confirming the presence of functional coreceptor on the BC7-CCR5 cells.

Neutralization escape

Neutralization escape is common during HIV-1 infection and poses a challenge for vaccines that aim to control rather than prevent infection (16). In subject AC10, low levels of virus replication might have given rise to an escape variant sometime after rebound viremia declined. To test this, virus was isolated at multiple time points and assayed with autologous serum samples obtained before and after isolation. Attempts to isolate virus from cryopreserved PBMC obtained 466, 667, and 734 days after treatment cessation were unsuccessful. This prompted us to use PBMC from fresh

Table III. *Rebound isolate AC10.44 requires CD4 for infection*

	ng p24/ml[a]			
	BC7-CCR5		PBMC	
Virus	Day 6	Day 12	Day 6	Day 12
AC10.44	<0.1	<0.1	80	32
NL-ADA	4	19	50	25

[a] Cultures of BC7-CCR5 (10^6 cells/ml) and mitogen-stimulated PBMC (5×10^6 cells/ml) were inoculated with cell-free virus and incubated overnight at 37°C. Cells were washed two times with growth medium, resuspended in 10 ml of growth medium, and incubated at 37°C. Media were replaced every 3 days. Culture fluids (125 μl) were collected at 6 and 12 days and mixed with an equal volume of 0.5% Triton X-100. Viral p24 concentrations were determined by Ag-specific ELISA (DuPont). BC7-CCR5 cells were maintained in growth medium containing puromycin (0.3 μg/ml) for stable CCR5 expression.

Table IV. *Neutralization escape in subject AC10*

Day of Serum Collection[a]	Neutralizing Ab Titer Against Virus Isolated on Day[b]			
	44	119	164	923
31	<4	<4	<4	<4
58	460	<4	<4	<4
93	350	<4	<4	<4
119	nt	<4	<4	<4
128	220	<4	<4	<4
143	155	<4	<4	<4
164	nt	<4	<4	<4
224	nt	9	17	<4
295	nt	54	35	<4
445	nt	48	282	<4
515	nt	110	>320	<4
667	nt	67	>320	<4
734	61	99	320	<4
825	nt	192	147	<4
923	306	137	299	22
970	310	182	182	<4

[a] Days after treatment cessation.
[b] Neutralization was measured in the fixed virus dose assay in PBMC with virus isolated at the indicated number of days after treatment cessation; nt, not tested.

heparinized blood drawn on day 923, which yielded isolate AC10.923. This isolate exhibited an R5 biologic phenotype and resisted neutralization (titer <4) by all earlier serum samples that neutralized the rebound isolate potently (Table IV). Isolate AC10.923 was neutralized by contemporaneous serum only, suggesting the isolate emerged earlier than day 923.

To determine how soon the virus escaped neutralization, attempts were made to isolate virus from cryopreserved PBMC obtained 119 and 164 days after treatment cessation; these are time points when minor increases in plasma viremia were observed. Both isolation attempts were successful and yielded viruses possessing an R5 phenotype. Each isolate resisted neutralization by contemporaneous and earlier serum samples but was highly sensitive to neutralization by later serum samples (Table IV). Results with AC10.119 indicated that escape occurred within 75 days from the time of AC10.44 isolation. Isolate AC10.923 was more resistant to neutralization than AC10.119, suggesting it was the product of one or more additional rounds of neutralization escape. It was not clear whether isolate AC10.164 represented one of those rounds of escape, as it was sensitive to neutralization by the same samples that neutralized AC10.119.

Full-length gp160s of all four isolates were cloned and sequenced (Fig. 5). Each gp160 molecule contained 27–29 potential sites for *N*-linked glycosylation, including a site (aa307) that is known to mask neutralization epitopes in V3 (66). The late escape variant, AC10.923, exhibited 27 amino acid changes in gp120 and 7 changes in gp41. Notably, clusters of amino acid changes were

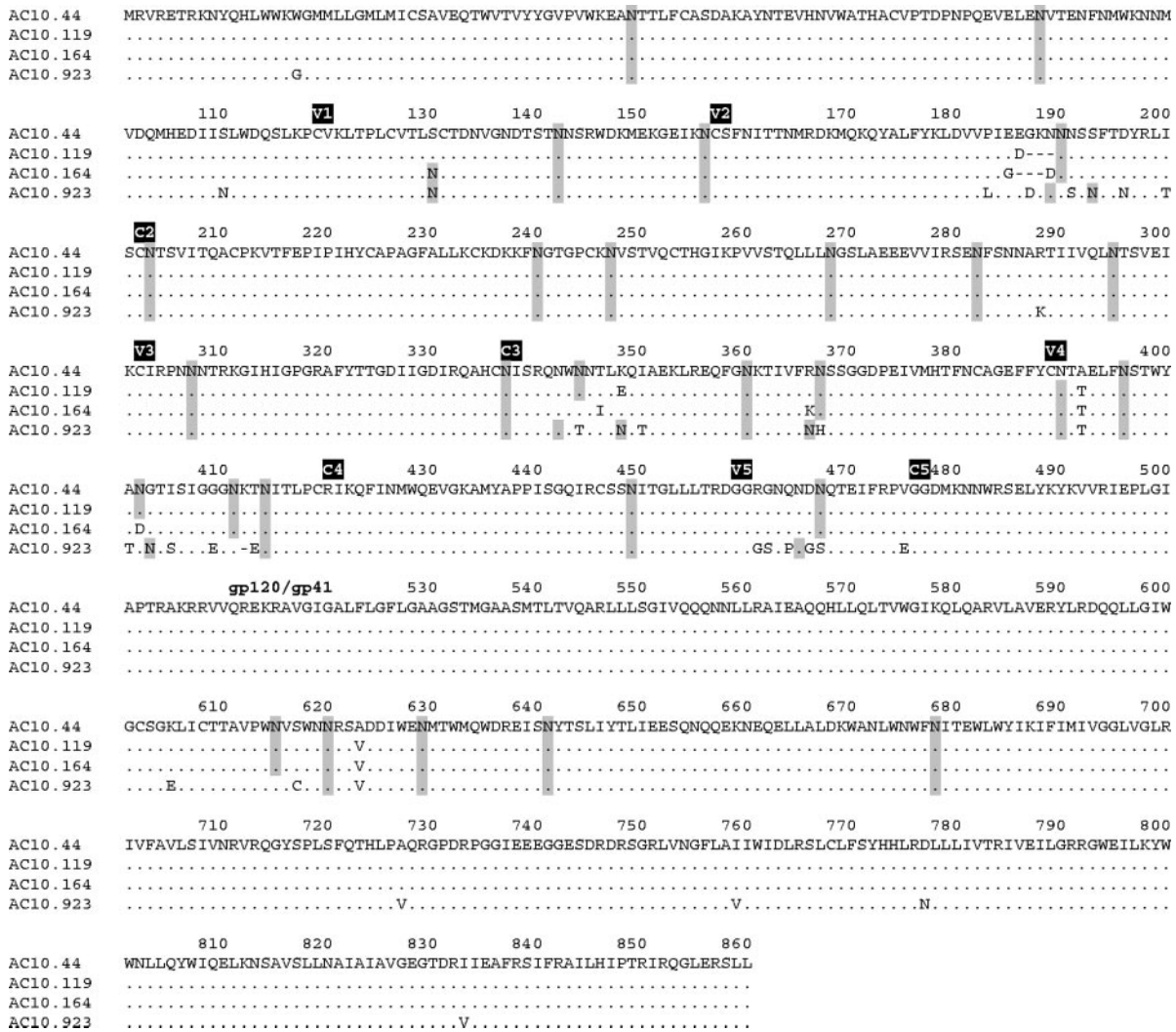

FIGURE 5. Comparison of the gp160 amino acid sequences of the rebound isolate AC10.44 with subsequent escape variants. Potential sites of *N*-glycosylation are shaded.

present in the V2, C3, V4, and V5 regions of gp120, suggesting foci of changes important to escape neutralizing Abs. A single amino acid deletion was detected at position 412 in V4. No amino acid changes occurred in V3. This escape variant gained a potential *N*-linked glycosylation site in each of the V1, V2, C3, and V4 regions of gp120, lost two sites (V4 and gp41) and showed a minor shift in position of one or two amino acids for an additional five sites in gp120 relative to the AC10.44 parent.

Fewer amino acid changes were present in escape variants AC10.119 and AC10.164. The most notable change was a three amino acid deletion in the V2 region of both variants. There were four additional amino acid changes in AC10.119 and eight amino acid changes in AC10.164 scattered throughout gp160. Notably, there were no changes in the V3 region of any escape variant, suggesting this region was not under selection pressure.

CD8$^+$ T cell response

Given the sustained control of viremia after neutralization escape, it was of interest to determine the status of this subject's virus-specific CD8$^+$ T cell response before and after the escape variant was isolated. ELISPOT assays were performed with a series of overlapping peptides spanning the entire HIV-1 genome as well as 28 optimal CTL epitope peptides described for the study subject's HLA class I type (A2/24 B38/60, Cw2/12). These assays detected a narrow and relatively weak CD8$^+$ T cell response directed against a total of four overlapping peptides as well as one described HLA-A24-restricted epitope in p17 Gag that was not detected using the corresponding overlapping peptide for the first 144 days (21 wk) after treatment cessation (Table V). The response broadened substantially thereafter, ultimately targeting at least 22 different epitopes, including several

Table V. *Virus-specific CD8$^+$ T cell response in subject AC10*

Peptide	Amino Acid Sequence	SFC/10^6 Cells at Day[a]													
		−3	44	58	119	144	227	295	445	515	667	734	825	873	923
Gag															
2	SGGELDRWEKIRLRPGGK	0	20	30	0	10	0	0	0	0	40	10	30	***260***	***170***
4	GKK**KYKLKHIVW**ASREL[b]	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11	TGSEELR**SLYNTVATL**Y[c]	0	20	40	0	10	0	0	***60***	***ND***	***ND***	***180***	***260***	***890***	***480***
12	**SLYNTVATL**CVHQRIEV[c]	0	10	30	0	0	20	0	***110***	***320***	***400***	***320***	***90***	***900***	***750***
24	PMFSAL**SEGATPQDL**NTM	0	0	0	0	0	20	0	0	30	0	0	20	0	30
28	LKETINEEAAEWDRLHPV	0	40	0	0	10	0	40	20	0	0	0	10	***170***	***330***
40	GPKEPFRDYVDRFYKTLR	10	0	10	0	10	40	20	***60***	ND	ND	***60***	***100***	20	0
58	QMKDCTERQANFLGKIW[c]	0	0	0	0	50	10	***80***	50	***220***	***240***	***300***	***130***	***450***	***310***
59	RQANFLGKIWPSHKGR[c]	0	20	10	0	0	***70***	***120***	***250***	***350***	***459***	***480***	***230***	***610***	***540***
63	TAPPEESFRFGEETTTPSQK	10	0	30	0	0	0	30	10	30	***60***	30	***200***	***500***	***490***
Nef															
71	DGVGAVSRDLEKHGAI	10	10	10	0	0	10	20	***180***	***320***	***180***	***280***	***100***	***280***	***180***
79	SHFL**KEKGGLEGL**IYSQK	10	20	20	0	0	10	***110***	***200***	***550***	***680***	***680***	***970***	***1420***	***1410***
84	NYTPGPGIRYPLTFGWCF[c]	10	10	20	0	20	10	0	20	0	***240***	***430***	***150***	***980***	***800***
85	RYPLTFGWCFKLVPV[c]	20	10	30	50	10	40	0	0	20	***400***	***590***	***350***	***1060***	***590***
88	EANEGENNSLLHPMSLH	0	50	50	40	20	50	***110***	0	***180***	***440***	***490***	***220***	***1100***	***1030***
90	SLHGMDDPEKEVLVWKF[c]	40	0	50	***60***	***90***	***160***	***370***	***260***	***390***	***370***	***320***	***250***	***600***	***520***
91	PEKEVLVWKFDSRLAFHH[c]	20	40	50	20	0	***180***	***300***	***270***	***360***	***410***	***380***	***150***	***350***	***340***
93	AFHHMARELHPEYYKDC	10	20	20	10	10	30	***170***	***80***	***420***	***410***	***440***	***310***	***850***	***540***
Pol															
152	GTVSESFPQITLWQRPLV	0	0	50	0	0	0	0	0	10	10	***90***	10	***410***	***390***
194	K**IEELRQHLL**RWGFTTPDK	0	10	0	0	20	0	0	0	0	0	0	0	40	20
271	YYRDSRDPLWKGPAKLLW	40	***110***	***240***	***240***	***260***	***470***	***740***	***660***	***690***	***770***	***740***	***810***	***1450***	***1310***
Vpr															
283	SLGQHIYETYGDTWAGV	***80***	***120***	***220***	***150***	***130***	***110***	***570***	***660***	***660***	***800***	***850***	***670***	***1110***	***940***
Env															
296	LFCASDACAYDTEVHNVW[c]	10	50	20	0	0	20	0	50	10	20	***80***	40	***510***	***390***
297	AYDTEVHNVWATHACV[c]	10	20	50	0	0	10	50	0	10	40	***160***	***130***	***420***	***430***
302	WKNNMVEQMHEDIISLW[c]	40	20	50	0	40	50	***70***	***280***	***800***	***960***	***770***	***820***	***1240***	***960***
303	QMHEDIISLWDQSLKPCV[c]	20	10	20	0	20	30	***80***	***140***	***690***	***680***	***820***	***310***	***870***	***650***
366	RVLAVE**RYLKDQQLL**GIW	10	0	0	0	0	0	0	30	0	30	***60***	***90***	***170***	***130***
374	NYTSLIYTLIEESQNQQEK	50	0	0	0	10	20	0	10	10	***70***	***70***	20	***230***	***180***
398	AVAEGTDRVIEVVQRACR	10	***80***	***70***	0	***80***	0	0	10	***300***	***420***	***210***	***80***	***170***	***270***
Vif															
413	VSIEWRKKRYSTQVDPDL[c]	10	30	10	0	10	20	0	0	10	***100***	***150***	***230***	***680***	***600***
414	RYSTQVDPDLADQLIHLY[c]	10	10	***70***	0	0	10	0	0	30	***150***	***230***	***230***	***360***	***550***
Optimal CD8 epitopes															
A2-SL9 (p17)	SLYNTVATL	10	10	0	0	0	0	***150***	***240***	***680***	***720***	***610***	***760***	***970***	***1110***
A24-RW8 (env)	RYLKDQQLL	10	10	30	0	20	0	0	0	0	0	50	***530***	***1200***	***130***
A24-KW9 (p17)	KYKLKHIVW	20	20	20	***80***	***90***	***130***	***340***	***140***	***ND***	***ND***	***ND***	***190***	***100***	***140***
B60-SL9 (p24)	SEGATPQDL	20	0	20	0	0	30	0	10	20	40	0	10	50	***80***
B60-IL9 (RT)	IEELRQHLL	0	0	0	20	0	10	0	0	0	10	20	20	***80***	10
B60-KL9 (Nef)	KEKGGLEGL	0	0	10	0	10	10	0	***280***	***0***	***ND***	***480***	***690***	***>1000***	***>1000***
Totals		80	310	600	530	650	1120	3210	3870	6858	8959	10270	9050	21390	17740

[a] IFN-γ-producing cells at the indicated number of days after treatment cessation. A total of 410 overlapping peptides (17–19 mers) spanning the entire expressed HIV-1 clade B consensus sequence as well as 28 peptides corresponding to optimal CTL epitopes described for the study subject's HLA class I type (A2/24 B38/60, Cw2/12) were tested. Only peptides that induced significant responses (>50 SFC/10^6 PBMC above background and three times above mean background activity) at least at one time point are shown (positive responses are in italics and boldface). Targeted described optimal CTL epitopes within overlapping peptides are shown in boldface type. nd, not determined.

[b] Overlapping peptides that contained described recognized optimal CTL epitopes, but were not recognized themselves, are shown. These responses represented low magnitude CD8$^+$ T cell responses only detectable using the optimal epitope sequence.

[c] Responses to two overlapping peptides may contain the same CD8$^+$ T cell epitope in their overlap and were therefore counted as one CD8$^+$ T cell response in the analysis.

optimal described epitopes in p17 Gag, p24 Gag, RT, Env, and Nef, at 873 and 923 days. The majority of targeted optimal CTL epitopes were not previously described, emphasizing the need for a comprehensive characterization of HIV-1-specific $CD8^+$ T cell responses. The magnitude of HIV-1-specific $CD8^+$ T cell responses directed against overlapping peptides increased significantly from 690 SFC/10^6 PBMC at day 144 to 11,970 SFC/10^6 PBMC at day 923 ($p < 0.00001$). The most potent $CD8^+$ T cell responses were directed against HIV-1 Gag, Nef, and Env peptides. Taken together, these data suggest that following treatment interruption HIV-1-specific $CD8^+$ T cell responses were initially weak and narrowly directed against a limited number of epitopes, but subsequently broadened significantly.

Discussion

Subject AC10 represented a rare opportunity to investigate the potential value of a rapid neutralizing Ab response after the cessation of early HAART. Neutralizing Abs from subject AC10 were capable of reducing the infectivity of his rebound virus by >4 logs in vitro and were present in full force at a time when the virus-specific $CD8^+$ T cell response was weak and narrow in specificity compared with other subjects in the study cohort (13). Abs with such high potency may be expected to have an impact in vivo, possibly accounting for the dramatic decline in rebound viremia after treatment cessation. Moreover, the rapid rise of those Abs to peak levels just 21 days after rebound viremia was first detected makes it possible that they limited both the magnitude of viral rebound and the extent of early immunologic damage caused by the virus.

Neutralizing Abs might not have acted alone in down-regulating the rebound in viremia, because virus-specific $CD8^+$ T cells were not totally absent at this time. However, the wide disparity in potency of measurable immune responses at this time suggests that neutralizing Abs played an important functional role. The same may be true for the small number of cases reported previously in which autologous neutralizing Abs correlated with at least temporary reductions in plasma viremia when subjects were either on HAART (67), intermittently nonadherent to HAART (68), or discontinued HAART (12). There also has been a case of a needlestick injury in a healthcare worker who exhibited autologous neutralizing Abs as peak viremia declined by ~2 logs during primary infection (69). A causal relationship between neutralizing Abs and viremia control has been suggested for a subset of HIV-1-infected long-term nonprogressors (10, 30, 37, 70) but the low viremia in those subjects made virus isolation difficult and the Abs were rarely tested against autologous virus. Also, the contribution of neutralizing Abs to long term nonprogression must be weighed against potent virus-specific $CD8^+$ T cell responses in those subjects (10, 11, 71). Passive immunotherapy with either hyperimmune globulin from HIV-1-infected individuals or human mAbs has been shown to have only marginal virologic and clinical benefits (72–75). Perhaps the clearest evidence that neutralizing Abs are functional in HIV-1-infected individuals comes from the emergence of neutralization escape variants (76–79).

We hypothesize that a rapid and potent neutralizing Ab response in subject AC10 resulted from earlier B cell priming, before viremia was controlled by HAART. In principle, similar priming by candidate vaccines would benefit therapeutic immunization and vaccines that aim to control rather than prevent infection. This will of course require an immunogen that targets a broad spectrum of primary HIV-1 isolates, which has yet to be identified. Nonetheless, in support of subject AC10 as a model case, the phenotype of rebound isolate AC10.44 was typical of most primary HIV-1 isolates. Thus, the virus was relatively insensitive to neutralization by a variety of reagents that possessed potent neutralizing activity against TCLA strains. Moreover, experiments with V3 peptides revealed the presence of linear epitopes on the virus that were poor targets for neutralization by autologous Abs. Finally, the isolate required CD4 for infection, implying that its gp120 did not exhibit a rare CD4-induced conformation that has been associated with high neutralization sensitivity (60–63). These combined results support the notion that isolate AC10.44 does not possess an unusual phenotype that might explain its potent neutralization by autologous serum.

The antiviral impact of a rapid and potent autologous neutralizing Ab response and a gradually increasing virus-specific $CD8^+$ T cell response in subject AC10 was not sufficient to prevent neutralization escape. Escape was associated with a growing number of mutations in gp160 over time, indicating active genetic evolution despite low plasma virus RNA loads below 1000 copies/ml. Escape was detected as soon as 75 days after the rebound virus was isolated. Virus-specific $CD8^+$ T cell responses had increased little by this time, yet plasma viremia remained low. Notably, anecdotal cases of viremia control and prolonged survival have been reported when neutralizing Abs, transferred passively, were present for only a short period of time during early infection with highly pathogenic strains of SIV and SHIV in macaques (24–29, 80). It is difficult to ascertain what immune effector mechanisms accounted for viremia control in subject AC10 after neutralization escape first occurred, which was before the $CD8^+$ T cell response increased significantly. This is a very curious phenomenon that deserves further study. As mentioned above, virus-specific $CD8^+$ T cells were not totally absent and might have played a partial role. Moreover, our peptides for detecting virus-specific $CD8^+$ T cells were not completely matched to the AC10 isolates, making it possible that we missed one or more key specificities. Other immune mechanisms might also have come into play, such as Ab-dependent cellular cytotoxicity and chemokine production (81). It is also possible that neutralizing Abs selected a variant strain that possessed an attenuated phenotype. Attenuating mutations that map to Env have been described (82).

What has emerged from these data is a profile of immune-mediated viremia control in an HIV-1-infected individual in which neutralizing Abs were an early dominant antiviral immune response. Those Abs might have provided time for other antiviral immune responses to mature to a level that was adequate to control the virus after neutralization escape occurred. Vaccine priming strategies that aim to augment the neutralizing Ab response that emerges after primary HIV-1 infection or treatment cessation might afford similar benefits. Continued follow-up of the immunologic, virologic, and clinical status of subject AC10, together with a similar evaluation of related subjects, will be necessary to gain further support for this hypothesis. Detailed molecular and immunologic studies of mAbs and cloned envelope glycoproteins from subject AC10 might provide insights into the nature of epitopes responsible for potent autologous neutralizing Ab responses and ways to generate those responses with candidate vaccines.

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