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Increased Neutralization Sensitivity of Recently Emerged CXCR4-Using Human Immunodeficiency Virus Type 1 Strains Compared to Coexisting CCR5-Using Variants from the Same Patient[▽]

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CXCR4-using (X4) human immunodeficiency virus type 1 (HIV-1) variants evolve from CCR5-using (R5) variants relatively late in the natural course of infection in 50% of HIV-1 subtype B-infected individuals and subsequently coexist with R5 HIV-1 variants. This relatively late appearance of X4 HIV-1 variants is poorly understood. Here we tested the neutralization sensitivity for soluble CD4 (sCD4) and the broadly neutralizing antibodies IgG1b12, 2F5, 4E10, and 2G12 of multiple coexisting clonal R5 and (R5)X4 (combined term for monotropic X4 and dualtropic R5X4 viruses) HIV-1 variants that were obtained at two time points after the first appearance of X4 variants in five participants of the Amsterdam Cohort Studies on HIV-1 infection and AIDS. Recently emerged (R5)X4 viruses were significantly more sensitive to neutralization by the CD4-binding-site-directed agents sCD4 and IgG1b12 than their coexisting R5 viruses. This difference was less pronounced at the later time point. Early (R5)X4 variants from two out of four patients were also highly sensitive to neutralization by autologous serum (50% inhibition at serum dilutions of >200). Late (R5)X4 viruses from these two patients were neutralized at lower serum dilutions, which suggested escape of X4 variants from humoral immunity. Autologous neutralization of coexisting R5 and (R5)X4 variants was not observed in the other patients. In conclusion, the increased neutralization sensitivity of HIV-1 variants during the transition from CCR5 usage to CXCR4 usage may imply an inhibitory role for humoral immunity in HIV-1 phenotype evolution in some patients, thus potentially contributing to the late emergence of X4 variants.

Entry of human immunodeficiency virus type 1 (HIV-1) into a host cell is mediated by binding of the viral envelope glycoprotein 120 (gp120) to CD4 and a coreceptor, of which CCR5 and CXCR4 are the most important on primary cells (7, 8, 9). Primary HIV-1 infections are generally established by R5 viruses, which remain present throughout the course of infection (27). In approximately 50% of therapy-naive individuals infected with subtype B HIV-1, X4 viruses evolve from R5 variants, preceding an increased $CD4^+$ T-cell decline and accelerated progression to AIDS (4, 24, 27, 30).

The relatively late appearance of X4 HIV-1 variants is poorly understood. R5 and X4 subtype B HIV-1 variants can genetically be distinguished by the absence or presence of a positively charged amino acid on positions 11 and/or 25 in the third variable loop (V3) of the gp120 envelope (10). In vitro experiments revealed that these mutations in V3 (6), as well as other single or double mutations in V3 and other domains of gp120 (2, 12), are sufficient to change coreceptor usage. However, in spite of the high mutation rate of HIV-1, X4 viruses do not evolve rapidly in vivo and not in all infected patients. Moreover, the earliest detectable X4 variants in vivo show more than only one or two amino acid substitutions compared to coexisting R5 variants (16), suggestive of compensatory mutations. These observations point towards the existence of selective pressure against X4 HIV-1 evolution, the exact nature of which remains to be established.

During the conversion of CCR5 to CXCR4 usage in vitro and in vivo, HIV-1 has to traverse a phase with reduced replicative capacity and less-efficient coreceptor usage (16, 22, 34), indicating that HIV has to overcome a significant genetic hurdle to evolve from an R5 to an X4 phenotype. In addition, HIV-1 phenotype conversion may be suppressed by host immunity. In a previous study, we demonstrated that X4 variants emerged only after CD4 counts had dropped below 400 cells/μl blood (15). X4 variants may thus be considered opportunistic viruses that emerge as a result of immune failure and subsequently give rise to an accelerated loss of $CD4^+$ T cells. Since the interaction between the viral envelope proteins and the host cell receptors may be prevented by neutralizing antibodies, humoral immunity could play a role in the evolution of HIV-1 coreceptor usage by differentially neutralizing R5 and X4 variants.

In our present study, we compared the neutralization sensitivities of clonal R5 and (R5)X4 (this term is used throughout the paper for both monotropic X4 and dualtropic R5X4) HIV-1 variants that coexisted in vivo and that were isolated from five individuals around the moment of the first appearance of X4 variants and at a later time point during symptomatic disease. We show that R5 and (R5)X4 variants have different susceptibilities to CD4-binding-site (CD4bs)-directed

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TABLE 1. Characteristics of R5 and (R5)X4 HIV-1 virus variants

Patient no. (sex[a]; seropositivity at entry)	Viruses early post-X4			Viruses late post-X4		
	Time since SC or entry (mo)[b]	Time since X4 variant detected (mo)	Coreceptor usage (*n*)	Time since SC or entry (mo)[b]	Time since X4 variant detected (mo)	Coreceptor usage (*n*)[c]
ACH0039 (M; no)	11.2	−4.9	R5 (1)	38.9	22.8	R5 (2), X4 (1)
	18.2	2.1	R5 (1), R5X4 (2)	51.2	35.0	R5 (1), X4 (3)
	21.1	5.0	R5 (2), R5X4 (1)			
ACH0171 (M; no)	61.0	−1.5	R5 (1), R5X4 (1)[c]	89.5	27.0	R5 (4), R5X4 (4)
	67.0	4.5	R5 (2), R5X4 (2)			
	73.0	10.5	R5 (1), R5X4 (1)			
ACH1120 (M; no)	53.3	6.0	R5 (4), R5X4 (2), X4 (2)	64.6	17.2	R5 (4)
				66.4	19.1	R5X4 (4)
ACH6052 (M; yes)	0[d]	Unknown[d]	R5 (3), X4 (5)	32.0	Unknown[d]	R5 (3), X4 (5)
ACH9012 (F; no)	NT[e]	NT	NT	3.5	3.5	R5 (3), X4 (3)

[a] M, male; F, female.
[b] Time since seroconversion (SC) or entry of patient into HIV$^+$ cohort.
[c] As determined with transfected U87 indicator cell lines. *n*, no. of clones. For each time point, the presence of CXCR4-using variants in a bulk virus culture was determined in the MT2 assay, in which CXCR4 usage of single virus isolates can be missed.
[d] ACH6052 was seropositive and carried X4 variants at the time of entry into the cohort.
[e] NT, not tested.

agents. For two out of four patients, the (R5)X4 variants isolated early after the appearance of X4 were potently neutralized by autologous serum. We postulate that the difference in neutralization by host humoral immunity may influence virus phenotype evolution in vivo in these patients. However, the absence of a detectable anti-X4 response in others argues against a major role for the humoral response in this process and indicates that other selective pressures are also involved.

MATERIALS AND METHODS

Patients and viruses. Patients ACH0039, ACH0171, ACH1120, and ACH6052 were homosexual male participants of the Amsterdam Cohort studies on HIV-1 and AIDS who all developed X4 variants during a progressive disease course. Patient ACH9012 (female) was infected after a deliberate injection of a few ml of blood drawn from an AIDS patient (36). All patients were infected with subtype B HIV-1. None of the participants ever received multidrug antiretroviral therapy. Biological virus clones from time points early and late after the first appearance of X4 variants were obtained as previously described (27). For patient ACH9012, viruses of one time point, 3.5 months after seroconversion, were used. Phylogenetic analysis of coexisting R5 and X4 variants (data not shown) in combination with the equal contributions of R5 and X4 variants to total cellular viral load in the donor (35) implicate that the length of the period of R5 and X4 coexistence was such that the time point of virus isolation from ACH9012 equals the time point late after X4 emergence in the other patients. The moment of seroconversion was calculated as the midpoint between the last seronegative visit and the first seropositive visit. Similarly, the moment of the first appearance of X4 viruses was calculated as the midpoint between the last MT2-negative visit and the first MT2-positive visit.

For all of the HIV-1 variants studied here, the ability to replicate in the MT2 cell line was considered evidence of CXCR4 usage. In addition, CXCR4 usage for all clones was confirmed in peripheral blood mononuclear cells (PBMC) from a healthy donor homozygous for the 32-bp deletion in the CCR5 gene ($CCR5^{\Delta/\Delta}$). For subjects ACH0039, ACH0171, ACH1120, and ACH6052, coreceptor usage was confirmed in U87 indicator cell lines expressing CD4 and either CCR5 or CXCR4. In previous studies, we determined that the replication of R5X4 and X4 virus variants of all patients in PBMC could be inhibited by AMD3100, indicating that these variants mainly use CXCR4 to enter these primary cells (13, 29). The sequences of the gp120 V3 domains of R5 and (R5)X4 variants from various time points have been determined, all showing the amino acid residues at positions 11 and/or 25 of the V3 domain that are commonly associated with an R5 or X4 virus phenotype (33; also data not shown). To prevent a change in sensitivity of the virus variants to neutralization during in vitro culture, a minimum number of passages of the viruses in PBMC was performed (1).

Primary cells. PBMC were isolated from buffy coats obtained from healthy seronegative blood donors by Ficoll-Isopaque density gradient centrifugation. Cells (5×10^6/ml) were stimulated for 2 days in Iscove's modified Dulbecco medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and phytohemagglutinin (PHA) (5 μg/ml). Subsequently, cells (10^6/ml) were grown in the absence of PHA in medium supplemented with recombinant interleukin-2 (20 U/ml, Chiron Benelux, Amsterdam, The Netherlands).

Neutralization assay. Viruses were tested for their relative neutralization sensitivities against recombinant soluble CD4 (sCD4) (Progenics, Tarrytown, NY) and the human monoclonal antibodies (MAbs) IGg1b12 (a kind gift from D. Burton), 2F5, 2G12, and 4E10 (Polymun Scientific, Vienna, Austria), autologous serum, and pooled serum from healthy uninfected individuals. From each virus isolate, a final inoculum of 10 50% tissue culture infective doses in a volume of 100 μl was incubated for 1 h at 37°C with increasing concentrations of the neutralizing agents. Subsequently, the mixtures of virus with sCD4 or antibodies were added to 10^5 PHA-stimulated PBMC derived from healthy blood donors. PBMC incubated with sera were washed after 4 h. On day 7, virus production in culture supernatants was analyzed by an in-house p24 antigen capture enzyme-linked immunosorbent assay (ELISA) (31). Experiments were performed in triplicate. The percent neutralization was calculated by determining the reduction in p24 production in the presence of the agent compared to that in the cultures with virus only. In experiments measuring the neutralization by autologous serum, the percentage of inhibition by pooled HIV-1-negative serum was subtracted from the neutralization obtained using patient serum samples. When possible, 50% inhibitory concentration (IC_{50}s) were determined by linear regression.

Statistical analysis. For analysis of the neutralization by sCD4 and monoclonal antibodies, IC_{50}s were evaluated per virus variant using the Mann-Whitney U test. Viruses with IC_{50}s of <0.2 or >12.5 were assigned a value of 0.1 or 12.5, respectively, for calculations and statistical analyses. IC_{50}s of the autologous serum neutralization were evaluated for each time point and viral phenotype using the Wilcoxon signed ranks test. For this test, the IC_{50}s for the two variants used per time point and phenotype were linked per serum sample to the IC_{50}s of two other virus isolates of another time point or phenotype. Viruses with IC_{50}s of <40 or $>1{,}280$ were assigned a value of 20 or 1,280, respectively.

RESULTS

Patients and viruses. Longitudinally obtained coexisting R5 and dualtropic R5X4 and X4 [(R5)X4] viruses from five participants of the Amsterdam Cohort Studies were available from previous studies (14, 33, 34, 36). From four patients infected via homosexual contact (ACH0039, ACH0171, ACH1120, and ACH6052), biological virus clones were isolated shortly after the first detection of X4 variants and at a later time point 2 years before (ACH0171) or during symptomatic disease (Table 1). Patient ACH9012 (female) was par-

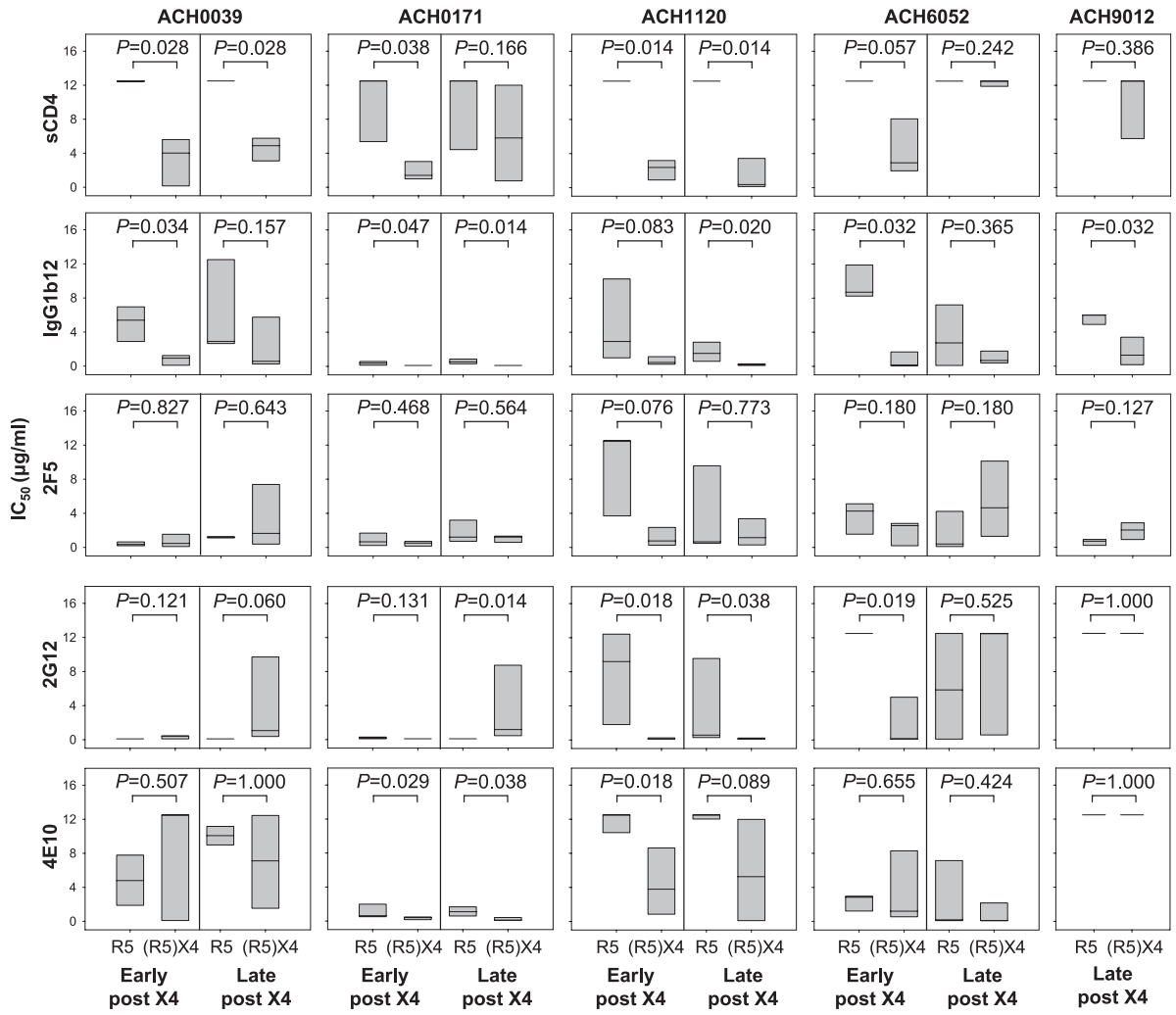

FIG. 1. Sensitivities for sCD4-, IgG1b12-, 2F5-, 2G12-, and 4E10-mediated neutralization of coexisting R5 and (R5)X4 HIV-1 obtained early or late after X4 emergence. Neutralization was determined by a 7-day culture of clonal virus isolates on PBMC in the presence or absence of serial dilutions of neutralizing agent, followed by analysis of p24 production by ELISA. Distribution of IC_{50}s for individual virus clones as determined by linear regression is shown. Bottom, middle, and top horizontal lines represent the 25th, 50th, and 75th percentiles, respectively. Per time point, IC_{50}s of R5 and (R5)X4 viruses were evaluated using the Mann-Whitney U test. Data shown are from one representative experiment out of two to three performed.

enterally infected with a mixture of R5 and X4 viruses that were first isolated 3.5 months after transmission (Table 1). Since X4 variants had already developed in the donor, the exact time since X4 appearance was not known, and these viruses were considered late post-X4 emergent (35) (see Materials and Methods).

Sensitivities of coexisting R5 and (R5)X4 variants to neutralization by CD4-binding-site-directed agents. The sensitivities of clonal coexisting R5 and (R5)X4 viruses to neutralization by sCD4 and the CD4-binding-site-directed MAb IgG1b12 were analyzed. R5 viruses obtained early after X4 emergence from all patients were resistant to sCD4 neutralization (IC_{50}s >12.5 μg/ml) (Fig. 1), while their coexisting (R5)X4 viruses were relatively susceptible (median IC_{50}s ranging between 1.40 and 4.02 μg/ml) (Fig. 1), although this approached significance only in patient ACH6052. In line with this observation, (R5)X4 variants obtained early after X4 emergence showed an increased sensitivity to IgG1b12 compared to their coexisting R5 variants, although this was not statistically significant for viruses from patient ACH1120 (Fig. 1).

For three out of five patients (ACH0039, ACH0171, and ACH1120), (R5)X4 viruses obtained late after X4 emergence still showed significantly higher sensitivities to neutralization by sCD4 and/or IgG1b12 than their coexisting R5 variants. For viruses isolated from the other patients, differential neutralization sensitivities were lost due to an increased susceptibility of the R5 viruses to IgG1b12 (although this reached statistical significance only for viruses from patient ACH6052 and not for those from patient ACH0039) and/or an increased resistance to sCD4 of the (R5)X4 variants (statistically significant for viruses from ACH6052 but not for viruses from ACH0171). X4 viruses from patient ACH9012 showed higher neutralization sensi-

TABLE 2. Autologous serum neutralization titers of early and late post-X4 virus clones of ACH0171

Clone	Time since X4 (mo)[a]	Phenotype	Neutralization titer (IC_{50}) at mo since X4[a,b]			
			−16.2	−7.5	10.5	27.0
171.31.ROF6	4.5	R5	125	79	69	87
171.33.ROA1	10.5	R5	87	84	81	114
171.41.ROE2	27.0	R5	<40	<40	<40	<40
171.41.ROC5	27.0	R5	<40	<40	<40	<40
171.31.ROB9	4.5	R5X4	>1,280	>1,280	>1,280	>1,280
171.33.ROA2	10.5	R5X4	151	768	343	NT
171.41.D12	27.0	R5X4	84	<40	82	<40
171.41.RAF5	27.0	R5X4	51	<40	<40	80

[a] Months since X4 variant was detected.
[b] Neutralization was determined by 7-day culture of clonal virus isolates on PBMC in the presence or absence of serial dilutions of serum, followed by analysis of p24 production by ELISA. IC_{50}s were determined by linear regression after subtracting the inhibition obtained using human pooled HIV-1-negative serum.

TABLE 4. Autologous serum neutralization titers of early and late post-X4 virus clones of ACH1120

Clone	Time since X4 (mo)[a]	Phenotype	Neutralization titer (IC_{50}) at mo since X4[a,b]			
			(−18.0)	(−8.7)	(6.0)	(17.3)
1120.53.1B6	6.0	R5	<40	<40	<40	<40
1120.53.1E4	6.0	R5	<40	<40	<40	<40
1120.57.3E7	17.3	R5	<40	<40	<40	52
1120.57.3C4	17.3	R5	<40	<40	<40	85
1120.254	6.0	X4	42	<40	<40	44
1120.255	6.0	X4	<40	<40	<40	41
1120.267	19.1	R5X4	<40	<40	<40	<40
1120.268	19.1	R5X4	<40	<40	<40	<40

[a] Months since X4 variant was detected.
[b] Neutralization was determined by 7-day culture of clonal virus isolates on PBMC in the presence or absence of serial dilutions of serum, followed by analysis of p24 production by ELISA. IC_{50}s were determined by linear regression after subtracting the inhibition obtained using human pooled HIV-1-negative serum.

tivities to IgG1b12 and equal resistance to sCD4 compared to those of the coexisting R5 variants.

Equal sensitivities of coexisting R5 and (R5)X4 variants to zidovudine excluded that the differences in neutralization sensitivity for sCD4 and IgG1b12 observed here were the result of differences in the replication rate between the various virus variants (data not shown).

Sensitivities of coexisting R5 and (R5)X4 HIV-1 variants to neutralization by non-CD4bs-directed agents. We next analyzed whether coexisting R5 and X4 variants also had differential susceptibilities to neutralization via epitopes outside the CD4-binding site. To this end we determined the sensitivities of the coexisting R5 and X4 viruses to neutralization by MAb 2G12 (recognizing a carbohydrate epitope on gp120) and monoclonal antibodies 2F5 and 4E10 (both binding to the membrane-proximal external region of gp41). For 2G12, increased neutralization sensitivity compared to that of their coexisting R5 variants was observed for early and late (R5)X4 viruses in patient ACH1120 and for the early (R5)X4 viruses in patient ACH6052 (Fig. 1). In contrast, late (R5)X4 viruses from patient ACH0171 were significantly more resistant to 2G12 neutralization than their coexisting R5 variants. For 4E10, early (R5)X4 viruses from patient ACH0171 and early and late (R5)X4 viruses from patient ACH1120 were more sensitive to neutralization than their coexisting R5 viruses (Fig. 1). No differences were observed for 2F5 (Fig. 1). Overall, some differences in neutralization susceptibilities were observed between R5 and (R5)X4 viruses for non-CD4bs-directed agents but not to the same extent as seen for sCD4 and IgG1b12.

TABLE 3. Autologous serum neutralization titers of early and late post-X4 virus clones of ACH6052

Clone	Time since entry (mo)[a]	Phenotype	Neutralization titer (IC_{50}) at mo since entry[a,b]		
			0	6.8	32.1
6052.1G2	0	R5	<40	<40	50
6052.1E2	0	R5	<40	<40	<40
6052.4C4	32.1	R5	<40	<40	<40
6052.4C3	32.1	R5	<40	<40	<40
6052.1H4	0	X4	>1,280	339	>1,280
6052.1G3	0	X4	>1,280	396	288
6052.4A10	32.1	X4	<40	<40	<40
6052.4B11	32.1	X4	<40	<40	<40

[a] Months since entry of patient into HIV^+ cohort.
[b] Neutralization was determined by 7-day culture of clonal virus isolates on PBMC in the presence or absence of serial dilutions of serum, followed by analysis of p24 production by ELISA. IC_{50}s were determined by linear regression after subtracting the inhibition obtained using human pooled HIV-1-negative serum.

Sensitivities of coexisting R5 and (R5)X4 HIV-1 variants to neutralization by autologous serum. The differential neutralization sensitivities of coexisting R5 and (R5)X4 variants might suggest that X4 variants can emerge only in the absence of neutralizing humoral immunity. To examine whether the humoral immune response indeed deteriorated around the time point of the appearance of X4, we analyzed the neutralization sensitivities of coexisting R5 and (R5)X4 viruses to autologous serum samples obtained before and after the first appearance of X4 variants. Since ACH6052 and ACH9012 carried X4 variants at the time of entry into the cohort, only serum samples obtained after X4 emergence were available from these patients. Unfortunately, due to limited amounts of serum, it was not possible to analyze all virus variants. We therefore randomly chose two virus variants per time point and phenotype for determination of neutralization by autologous serum. Early (R5)X4 variants from patients ACH0171 and ACH6052 were highly sensitive to neutralization by sera from all time points (IC_{50} titers, >200) (Tables 2 and 3), which was significantly different from neutralization of the early R5 variants (for ACH0171, $P = 0.018$; for ACH6052, $P = 0.027$), as well as the late X4 variants (for ACH0171, $P = 0.018$; for ACH6052, $P = 0.026$). Virus variants from patients ACH0039, ACH1120, and ACH9012, irrespective of phenotype or time point of isolation, either were not neutralized or were neutralized at relatively low serum dilutions (Table 4, Table 5 and Table 6). No differences between virus variants from different time points or in viral tropism were observed for these patients ($P > 0.050$).

DISCUSSION

We compared the neutralization sensitivities of coexisting R5 and (R5)X4 viruses isolated early and late after X4 emergence. In a previous study, we (11) and others (3, 17, 20, 32)

TABLE 5. Autologous serum neutralization titers of early and late post-X4 virus clones of ACH0039

Clone	Time since X4 (mo)[a]	Phenotype	Neutralization titer (IC_{50}) at mo since X4[a,b]			
			−9.4	−2.8	7.1	37.1
39.18.E11	−4.9	R5	<40	<40	<40	<40
39.21.D5	5.0	R5	<40	173	62	55
39.20.1E10	2.1	R5X4	82	169	197	277
39.21.H10	5.0	R5X4	<40	177	<40	60
39.28.A9	22.8	X4	<40	<40	<40	<40
39.*1.C4	35.0	X4	<40	93	222	<40

[a] Months since X4 variant was detected.
[b] Neutralization was determined by 7-day culture of clonal virus isolates on PBMC in the presence or absence of serial dilutions of serum, followed by analysis of p24 production by ELISA. IC_{50}s were determined by linear regression after subtracting the inhibition obtained using human pooled HIV-1-negative serum.

TABLE 6. Autologous serum neutralization titers of early and late post-X4 virus clones of ACH9012

Clone	Time since entry (mo)[a]	Phenotype	Neutralization titer (IC_{50}) at 3.5 mo since entry[a,b]
9012.A10	3.5	R5	<40
9012.A2	3.5	R5	<40
9012.A7	3.5	R5	<40
9012.E6	3.5	X4	<40
9012.E10	3.5	X4	<40
9012.F3	3.5	X4	<40

[a] Months since entry of patient into HIV^+ cohort.
[b] Neutralization was determined by 7-day culture of clonal virus isolates on PBMC in the presence or absence of serial dilutions of serum, followed by analysis of p24 production by ELISA. IC_{50}s were determined by linear regression after subtracting the inhibition obtained using human pooled HIV-1-negative serum.

reported no difference in neutralization sensitivities between R5, X4, or dualtropic R5X4 HIV-1 from different patients. However, even if differences among the various viruses had been observed, these most likely would have reflected interpatient variability of the neutralization sensitivities of unrelated HIV-1 variants rather than differences determined by coreceptor usage. In our present study, we therefore compared R5 and (R5)X4 virus clones that had been isolated from the same patient at the same time points.

Our data show that (R5)X4 viruses obtained early after X4 conversion are more sensitive to CD4-binding-site-directed agents than their coexisting R5 variants, whereas this differential sensitivity was less pronounced at a later time point. Since the differential sensitivity for non-CD4-binding-site-directed antibodies was not a general phenomenon among the five patients included in this study, mutations in the (co)receptor binding site associated with evolution from CCR5 to CXCR4 usage most likely change the neutralization phenotype in this region but do not change the general envelope conformation. In addition, coexisting dualtropic R5X4 and X4 viruses early after X4 emergence, although available from only one patient, had comparable neutralization sensitivities (data not shown). We therefore propose that the acquisition of CXCR4 usage, rather than the loss of CCR5 usage, coincides with changes in gp120 that increase neutralization sensitivity. In line with this is an observation by Lusso et al. (19), who showed that X4 viruses constitutively expose the epitope for monoclonal antibody D19, whereas R5 viruses are neutralized by this antibody only after pretreatment with sCD4, which would indeed imply a difference in conformation between R5 and X4 variants.

The neutralization-sensitive conformation of the viral envelope during the evolution from an R5 phenotype to an X4 phenotype would suggest that transition is possible only when an efficient neutralizing-antibody response is absent or lost due to immune deterioration associated with disease progression. However, for two out of four patients, the early (R5)X4 variants were efficiently neutralized by autologous serum, suggestive for the presence of neutralizing antibodies in vivo. Late (R5)X4 variants from these patients were not neutralized, indicating that (R5)X4 viruses had evolved towards increased neutralization resistance. This is most clearly demonstrated with patient ACH0171, from whom the earliest (R5)X4 virus, isolated 4.5 months after X4 appearance, was neutralized very efficiently (IC_{50} titers of >1,280). A virus isolated 10.5 months after X4 appearance was partially adapted to the autologous antibody response (IC_{50} titers between 151 and 768), whereas the late viruses, isolated 27.0 months after X4 appearance, were almost completely resistant to autologous serum neutralization. We observed differential autologous neutralization only between recently emerged (R5)X4 viruses and their coexisting R5 variants. The use of X4 and R5 viruses from later time points in infection may explain the lack of differential neutralization by serum between R5 and X4 viruses, as observed in a previous study by others (28).

Neutralizing antibodies present in serum obtained at a certain time point in infection generally neutralize viruses found during earlier time points (25, 37). Continuous viral escape makes the neutralization of HIV-1 by contemporaneous serum a rare event (25, 37). It is therefore not surprising that the R5 and (R5)X4 virus variants that were isolated late after X4 emergence were not neutralized by serum obtained at the earlier time points. However, we had not expected that R5 viruses isolated early after X4 emergence would resist neutralization by serum obtained later in infection. This may point to a decreasing neutralizing humoral immune response late in infection.

We were also surprised that the (R5)X4 viruses isolated shortly after the first appearance of X4 variants in patient ACH0171 were potently neutralized by serum samples obtained at earlier time points, even before the appearance of X4 viruses. Possibly, the conformational requirements of an early X4 envelope led to the exposure of certain neutralization epitopes that were initially exposed on R5 trimeric envelopes before the R5 viruses escaped from the antibodies directed against these epitopes. Alternatively, early X4 viruses might expose epitopes that are vulnerable to antibodies directed against other envelope structures, such as monomeric gp120, and initially have an oligomeric envelope conformation that resembles the oligomeric gp120 of neutralization-sensitive T-cell-line-adapted HIV-1. Indeed, a large proportion of the antibody repertoire against HIV-1 envelope protein is directed against nonneutralizing epitopes on shed, misfolded, or otherwise nonfunctional forms of gp120 (21, 26), which could explain why such antibodies are already present before X4 viruses have emerged. Another possibility for the presence of

antibodies directed against epitopes present on early X4 variants is that the virus has evolved towards CXCR4 usage earlier during infection but was previously inhibited by a potent humoral immune response. As a result of a decrease in selection pressure, the X4 variants may have been selected from the PBMC archive.

For three out of five patients, we did not observe effective neutralization of the early X4 viruses by serum samples from any time point. It has previously been shown that some patients fail to develop a humoral immune response against HIV-1 (25), which may explain the lack of neutralization observed in our study. Indeed, serum of patient ACH1120, which did not show neutralization of autologous virus, also did not contain broadly neutralizing antibodies (data not shown). In contrast, serum from ACH0171 displayed potent autologous neutralization as well as broadly neutralizing activity (data not shown). Patient ACH0039 rapidly progressed to AIDS (in 3.2 years after seroconversion), and may not have developed an effective immune response against HIV-1. As described above, patient ACH9012 became infected with a mixture of R5 and X4 variants, which prevented study of the role of autologous neutralization sensitivity in R5-to-X4 evolution. Interestingly, the proportion of X4 variants in serum in this patient increased preseroconversion but sharply decreased postseroconversion (5), suggestive for selective suppression of X4 variants by humoral immunity, although serum from ACH9012 did not show autologous neutralization. However, HIV-1 variants and the only serum from this patient were both obtained 3.5 months after seroconversion, again underscoring that neutralization of virus by contemporaneous serum is not commonly observed.

The observation that the emergence of X4 viruses is not prevented by the presence of neutralizing antibodies indicates that these viruses most likely appear in body compartments with lower antibody pressure than that in plasma. On the other hand, the early X4 variants used in this study have been isolated from PBMC, indicating that neutralization-sensitive X4 viruses were able to replicate in an environment containing neutralizing antibodies. The fact that X4 viruses are not hampered by the presence of neutralizing antibodies might suggest that these variants spread via direct cell-to-cell transmission, which limits the possibility of antibody neutralization.

In conclusion, we have shown here that early X4 variants are more susceptible to antibody neutralization than their coexisting R5 variants and that some patients mount a potent humoral immune response against these CXCR4-using variants. Although the humoral immune response in these patients was not sufficient to prevent the appearance of X4 variants, strong humoral immunity in certain patients could thus contribute to the inability of the virus to evolve to X4 usage. In this light, the much lower X4 conversion rate of subtype C HIV-1 (23) could be causally related to the stronger autologous neutralizing antibody titer for subtype C HIV-infected individuals (18). However, the observation that an anti-X4 response is absent for the other patients included in this study indicates that evolution of the virus phenotype may also be influenced by factors other than autologous humoral immunity.

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