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Coevolution of RANTES Sensitivity and Mode of CCR5 Receptor Use by Human Immunodeficiency Virus Type 1 of the R5 Phenotype

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The evolution of human immunodeficiency virus type 1 (HIV-1) coreceptor use has been described as the acquisition of CXCR4 use linked to accelerated disease progression. However, CXCR4-using virus can be isolated only from approximately one-half of individuals with progressive HIV-1 disease. The other half continue to yield only CCR5-using viruses (R5 phenotype) throughout the course of disease. In the present work, the use of receptor chimeras between CCR5 and CXCR4 allowed us to study the evolution of HIV-1 with the R5 phenotype, which was not revealed by studies of wild-type coreceptor use. All together, 246 isolates (173 with the R5 phenotype) from 31 individuals were tested for their ability to infect cells through receptor chimeras. $R5^{narrow}$ virus was able to use only wild-type CCR5, whereas $R5^{broad(1)}$ to $R5^{broad(3)}$ viruses were able to use one to three chimeric receptors, respectively. Broad use of chimeric receptors was interpreted as an increased flexibility in the mode of receptor use. $R5^{broad}$ isolates showed higher infectivity in cells expressing wild-type CCR5 than $R5^{narrow}$ isolates. Also, the increased flexibility of $R5^{broad}$ isolates was concomitant with a lower sensitivity to inhibition by the CC chemokine RANTES. Our results indicate a close relationship between HIV-1 phenotypic changes and the pathogenic process, since the mode and efficiency of CCR5 use as well as the decrease in the RANTES sensitivities of isolated viruses are significantly correlated with $CD4^+$-T-cell decline in a patient. One possible explanation is that ligand competition at the CCR5 receptor or changed CCR5 availability may shape the outcome of HIV-1 infection.

The severity of human immunodeficiency virus type 1 (HIV-1) infection varies dramatically among different individuals. Many factors, including age, associated diseases, immune activation, viral load, and viral phenotype, have been shown to affect disease progression (6, 11, 37, 43). Conceivably, the final disease outcome is the result of interplay between several or all of these factors. Here we focus on viral phenotype, specifically, its changes over time, and consider mechanisms by which phenotypic evolution might be regulated.

One important phenotypic trait linked to HIV-1 pathogenesis is the type of coreceptor used by HIV-1 to enter cells (7, 8). The coreceptor is a chemokine receptor, CCR5 or CXCR4 (2, 12, 14, 18, 19, 24), which together with CD4 allows efficient uptake of HIV-1 into cells. CCR5-using viruses (the R5 phenotype) are present throughout all stages of HIV-1 infection (10, 13, 25–27, 33, 41, 42, 51, 62, 63, 65). The evolution of HIV-1 coreceptor use during progressive disease has been extensively studied in terms of both viral genetics and phenotype and involves change from CCR5 use to CXCR4 use, alone (the X4 phenotype) or in combination with the use of CCR5 (the R5X4 phenotype) and/or other less significant coreceptors (multitropic viruses) (10, 25, 28, 31, 51–53, 55). CXCR4 use is linked to an increased virulence of HIV-1, and CXCR4-using viruses can be isolated prior to or during progression to AIDS (32, 35, 57).

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Even if the emergence of CXCR4-using HIV-1 has been linked to disease progression, it is isolated only from about one-half of AIDS patients (32, 35). Thus, progression to immunodeficiency may also occur in individuals in the absence of detectable CXCR4-using virus isolates. In these individuals the HIV-1 viral phenotype remains CCR5 dependent during the entire disease course (15, 29). However, R5 viruses from some AIDS patients develop increased resistance to inhibition by RANTES, i.e., the CC chemokine and natural ligand of CCR5 (29, 30, 34). It has been demonstrated that evolution to altered CCR5 usage can be induced in vitro, by exposing HIV-1 with the R5 phenotype to a small-molecule CCR5 antagonist (58). Recently, it was also shown that in individuals with progressive HIV-1 infection, R5 virus evolves to be more cytopathic over time, whereas this evolution is not seen in long-term asymptomatic individuals, indicating that this increasing cytopathicity of HIV-1 correlates with disease progression (36). Also, studies of macaques infected with different R5 clones implied that the fusogenicity of HIV-1 envelope is important to disease progression (21, 22). Thus, these observations suggest that R5 virus interaction with CCR5 may be altered both in vivo and in vitro.

In an attempt to document HIV biological variability during the pathogenic process, chimeric receptors between CCR5 and CXCR4 were used. Starting with the N terminus, parts of CCR5 were replaced with corresponding parts of CXCR4 (4). That study indicated that virus isolates from different individuals use CCR5 in different ways (33). In particular, the results hinted at differences in chimeric receptor use among viruses with the R5 phenotype. We therefore undertook a methodical

study of sequentially collected HIV-1 isolates with the R5 phenotype and explored their ability to use chimeric receptors. Using a series of chimeric receptors as instruments, we here describe the in vivo evolution of the R5 phenotype to a phenotype in which the CCR5 receptor is used more flexibly and more efficiently. With parallel changes in the mode of CCR5 receptor use, isolated viruses become more resistant to inhibition by the CC chemokine RANTES. In association with declining $CD4^+$-T-cell counts, changes in the mode of CCR5 use and the RANTES sensitivity of isolated viruses can be linked to pathogenesis.

MATERIALS AND METHODS

Patients and virus isolates. The 31 patients studied were selected from a larger cohort of 53 HIV-1-infected individuals described previously (29, 32, 33). The patients were adult homo- or bisexual men living in Sweden whose median follow-up period was 103 months; follow-up included the counting of CD4 cells and viral isolations, and since 1996 plasma viral RNA loads have been determined at the South Hospital in Stockholm, Sweden. For the present study, the patients were selected on the basis of different rates of CD4 decline in the first 5 years of their infections and the difference in their virus biological phenotypes as assayed by syncytium induction on MT-2 cells and by coreceptor use on U87.CD4 and GHOST (3) cells. Accordingly, patients could be divided into two groups. Seventeen patients yielded non-syncytium-inducing virus that used CCR5 (the R5 phenotype) throughout the observation period (64 isolates). Fourteen patients initially yielded virus with the R5 phenotype but that later switched to CXCR4 use (also called syncytium-inducing viruses). In the patients with viruses that switched phenotypes, we tested 109 R5 isolates preceding the acquisition of CXCR4 use and, following the switch, 73 CXCR4-using isolates (the X4 and R5X4 or multitropic phenotypes).

Viruses were isolated from peripheral blood mononuclear cells (PBMC) by a standard procedure (50), and coreceptor use of sequential isolates was determined (29, 33). The evolutionary relationship between virus isolates of the same patients was studied by phylogenetic analysis of V3 sequences as previously described (39). Virus stocks were prepared by infecting 6×10^6 to 8×10^6 phytohemagglutinin (PHA) (Boule, Stockholm, Sweden)-stimulated PBMC from two healthy donors with 1.5 ml of supernatant from infected PBMC. Supernatants were harvested on days 7 and 10 or 11 after infection and stored at −80°C. The medium used was RPMI medium (Invitrogen, Lidingö, Sweden) containing 10% fetal calf serum (Invitrogen), 50 U of penicillin (Invitrogen) per ml, 50 U of streptomycin (Invitrogen) per ml, 2 μg of Polybrene (Sigma, Stockholm, Sweden) per ml, and 10 U of interleukin-2 (IL-2) (Amersham Pharmacia, Uppsala, Sweden) per ml.

Cell lines. Human glioma U87.CD4 cells, stably expressing CD4 and one of the chemokine receptors CCR5 and CXCR4, were previously described (14). Cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal calf serum (Invitrogen) and antibiotics. Cultures were incubated in a humidified atmosphere with 5% CO_2 at 37°C, detached by treatment with 5 mM (pH 8.0) EDTA (VWR International, Lund, Sweden), and subdivided 1:2 to 1:4 twice a week.

Chimeric receptors were constructed by replacing successively increasing portions of CCR5 with corresponding regions of CXCR4 by a modification of the single-overlap and extension PCR approach (4). The resulting six chimeric constructs and the wild-type receptors were stably expressed in U87.CD4 cells. Proper surface expression of the receptors was secured by flow cytometry with a monoclonal antibody (55B5 [4]) raised against CXCR4 and recognizing the N terminus of the receptor. The U87.CD4 cell lines carrying chimeric receptors were cultured in the presence of 300 μg of Geneticin (Invitrogen) per ml and 0.5 μg of puromycin (Sigma) per ml. Geneticin selects for cells expressing CD4, and puromycin selects for cells expressing the chemokine receptors. Parental U87.CD4 cells, engineered to express CD4 but no chemokine receptor, were also included in the experiments.

Infection of U87.CD4 cell lines. Cells in 500 μl of medium per well were seeded into 48-well plates 1 or 2 days prior to infection to obtain a 50%-confluent cell layer by the time of infection. U87.CD4 cells with wild-type receptors were seeded in the same medium as described above for culturing. U87.CD4 cells with chimeric receptors were seeded in growth medium devoid of Geneticin and puromycin. When we infected the cells, the medium was removed and virus (at least 2 ng of the p24 antigen per ml according to an in-house p24 enzyme-linked immunosorbent assay [ELISA]) (56) was added to duplicate wells in a volume of 200 μl/well. Two hours after infection, medium with 2 μg of Polybrene (Sigma) per ml was added up to 500 μl/well. After an overnight incubation, cells were washed with PBS (0.12 M NaCl, 0.03 M phosphate [pH 7.2]), 1 ml of medium with Polybrene was added to each well, and the plates were further incubated. The cultures were kept for 7 days, and inspection for syncytium formation was performed daily. The supernatant for viral antigen detection by ELISA was removed on day 1, after being washed, and at the last day of infection. All supernatants were tested by the in-house p24 ELISA, and p24 production in cultures of cells expressing FC-1, FC-2, and FC-4b was also tested, using the commercially available Vironostika HIV Uni-Form II Ag/Ab Microelisa system (Biomérieux, Boxtel, The Netherlands), according to the protocol provided by the supplier. Viral antigen production was considered positive in the ELISA when the increase in absorbance between days 1 and 7 exceeded 0.2 in the in-house ELISA and 0.5 in the commercial ELISA.

Virus titration on U87.CD4 cells. Virus titration by hematoxylin staining of U87.CD4 cells was as previously described (54). Briefly, U87.CD4-CCR5 cells were infected as described above, but with fivefold virus dilutions in triplicate wells, starting with 14 ng of p24 per ml, according to the protocol of the commercially available Vironostika HIV-1 antigen ELISA (Biomérieux, Boxtel, The Netherlands). The day after infection, the plates were washed once with PBS and 1 ml of new medium was added. On day 4, the cell monolayers were washed once with PBS and fixed with methanol-acetone (1:1) for 5 to 10 min. For hematoxylin staining, which visualizes cell nuclei, the fixed cells were incubated for 2 to 3 min with hematoxylin (Merck, Darmstadt, Germany), washed with tap water, and dried. The number of plaques (distinct groupings of syncytial cells) was counted under a light microscope. Virus titers were calculated as numbers of PFU per milliliter with the following equation: (average number of plaques in triplicate wells × virus dilution)/volume in the well.

RANTES inhibition assay. RANTES inhibition of HIV-1 replication in PBMC cultures was done as described previously (29). In brief, PHA-stimulated donor PBMC (10^5 cells) were infected with HIV-1 primary isolates in the presence or absence of RANTES serially diluted in threefold steps starting from a final concentration of 600 ng/ml. At day 3 postinfection, the medium was changed and the RANTES content was restored. Supernatants were harvested at day 7 postinfection, and the 50% RANTES inhibitory concentration (IC_{50}) was analyzed by determination of the HIV-1 p24 antigen concentration by an ELISA, the Vironostika HIV Uni-Form II Ag/Ab Microelisa system (Biomérieux, Boxtel, The Netherlands).

Statistical analyses. The nonparametric Kruskal-Wallis test was used to compare groups of virus isolates with different R5 phenotypes {narrow ($R5^{narrow}$), broad with the use of one to three chimeric receptors [$R5^{broad(1)}$, $R5^{broad(2)}$, and $R5^{broad(3)}$]} based on sensitivity to RANTES and $CD4^+$-T-cell counts at the time of virus isolation. To demonstrate evolution within the R5 phenotype over time, the first and last isolates from 26 patients were compared by the Wilcoxon signed-rank test. The same test was used to compare the infectivities of the different groups of R5 isolates. Use of the FC-4b chimeric receptor and the frequencies of different R5 phenotypes in different patient groups were compared by chi-square analysis. Spearman rank correlation was used to show an association between the CD4-T-cell count at the time of isolation and RANTES sensitivity (expressed as the IC_{50}). Bivariate analysis was performed with a logistic regression model by calculating the odds ratio (OR) and its associated confidence-related interval (CI). In this model, CD4 status was treated as a categorical variable.

RESULTS

Defining the HIV-1 phenotype according to wild-type and chimeric coreceptor use. With the aim of studying a possible linkage between HIV pathogenesis and virus evolution in the context of host cell entry, we studied HIV-1 primary isolates obtained sequentially from 31 individuals with various levels of disease progression (a total of 246 isolates). In order to evaluate virus-coreceptor interactions, these isolates were tested on indicator U87.CD4 cells expressing CCR5, CXCR4, or one of the chimeric receptors FC-1, FC-2, FC-4b, FC-5, FC-6, and FC-7. As described previously (4), these chimeric receptors are hybrids of CCR5 and CXCR4 in which successively larger parts of CCR5 have been systematically replaced with corresponding parts of CXCR4 (Fig. 1). CXCR4-using viruses (with the X4, R5X4, and multitropic phenotypes) infected cells expressing

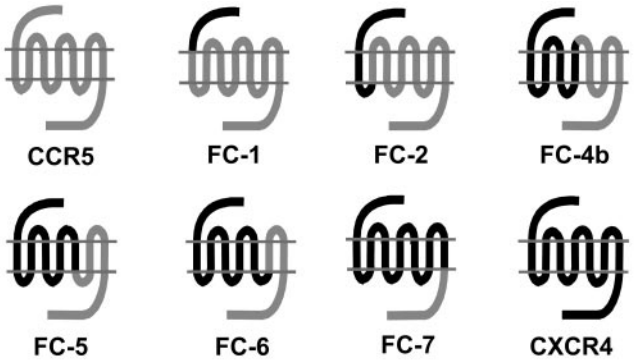

FIG. 1. Schematic pictures of the chemokine receptors CCR5 and CXCR4 and the chimeric receptors FC-1, FC-2, FC-4b, FC-5, FC-6 and FC-7, where successively larger parts of CCR5 have been replaced with corresponding regions of CXCR4.

all the different chimeric receptors, i.e., FC-4b, FC-5, FC-6, and FC-7, with the exception of FC-1 and FC-2 (33) (Table 1 and data not shown). Analysis of R5 isolates revealed that these viruses to various degrees used FC-1, FC-2, and FC-4b chimeras but did not use any other chimeric receptors or CXCR4. None of the viruses replicated in the parental U87.CD4 cells.

On the basis of chimeric receptor use, R5 viruses could be subdivided into two groups: those with the $R5^{narrow}$ phenotype and those with the $R5^{broad}$ phenotype. The $R5^{narrow}$ phenotype is shown by viruses that use wild-type CCR5 but no chimeric receptors, whereas viruses using at least one chimeric receptor in addition to CCR5 are designated $R5^{broad}$ viruses. Depending on the number of chimeric receptors used, the $R5^{broad}$ viruses could be further divided into $R5^{broad(1)}$, $R5^{broad(2)}$, and $R5^{broad(3)}$ phenotypes. The 38 isolates in the $R5^{broad(1)}$ category used either FC-2 or FC-4b (71 or 29%, respectively). Among the 64 isolates in the $R5^{broad(2)}$ category, the combination of FC-2 and FC-4b was the most frequent (77%). As previously described (33), the FC-1 chimera appeared to have the most restricted coreceptor function, since few of the isolates that were able to use the FC-2 or FC-4b receptor could use FC-1 for cell entry. Restriction is likely to be the result of an altered receptor conformation structure in which only the external part of the CXCR4 N terminus is anchored to CCR5, as with FC-1 but not with FC-2, as earlier suggested (4, 33).

The results indicate that HIV-1 isolates with the R5 phenotype may differ in their modes of CCR5 coreceptor use. Some viruses require the N terminus of CCR5 ($R5^{narrow}$), while others can bind to the coreceptor without the CCR5 N terminus ($R5^{broad}$). Within the latter group, exchange of the first extracellular loop of CCR5 to CXCR4 introduces an alternative receptor conformation. In spite of this, some of the R5 viruses can utilize chimeric receptor FC-4b when only the second and the third extracellular loops are derived from CCR5. Thus, these isolates appear not to depend on the N terminus and the first extracellular loop of CCR5 for host cell entry. However, the third extracellular loop of CCR5, represented by chimeras FC-5 and FC-6, is not enough to allow infection by any of the 173 R5 viruses that thus far have been tested (reference 33 and data not shown), indicating that these viruses depend on the second extracellular loop of CCR5 for infection.

The evolution of the R5 phenotype is related to pathogenesis. The first question we asked was whether the $R5^{narrow}$ and $R5^{broad}$ phenotypes indicate evolutionary steps of R5 virus. The phenotypes of the first and last R5 isolates from 26 patients (patients with two R5 isolates obtained at least 18 months apart) were compared (Table 2). Sixteen patients showed changes over time as follows: $R5^{narrow} \rightarrow R5^{broad(1)} \rightarrow R5^{broad(2)} \rightarrow R5^{broad(3)}$. These results established that the increasing capacity to use chimeric receptors indicates the evolution of the mode of CCR5 receptor use ($P = 0.004$, Wilcoxon signed-rank test). Thus, the phenotypic changes resulting in a broader ability to use chimeric receptors indeed represent sequential steps in R5 virus evolution.

Next we asked whether the R5 phenotype correlated with the degree of immune suppression of the patients from whom the virus was isolated. The results showed that viruses with the $R5^{broad(3)}$ phenotype were more often isolated from patients with low CD4 counts than from patients with high CD4 counts (Fig. 2). For the sake of statistical analysis, two isolates from each patient (the first and last as presented in Table 2) were considered ($P = 0.04$, Kruskal-Wallis test). For further analysis, our patient group of 31 HIV-1-infected individuals was divided into two groups on the basis of the phenotypes of viruses isolated: 17 patients retained virus with the R5 phenotype throughout the study period, and 14 patients acquired CXCR4-using virus. Accordingly, R5 viruses derived from these two groups of patients were designated R5 nonswitch and R5 switch viruses, respectively. When these two groups

TABLE 1. Use of chimeric receptors FC-1, FC-2, and FC-4b

Viral phenotype[a]	No. of isolates	% of replicating isolates using:					
		FC-1		FC-2		FC-4b	
		p24 only[b]	p24 + sync[c]	p24 only	p24 + sync	p24 only	p24 + sync
R5 nonswitch	64	20	2	3	63	22	17[d]
R5 switch	109	37	8	6	79	17	63
CXCR4 using	73	14	51	7	89	0	100

[a] R5 nonswitch, R5 isolates from patients with CCR5-using virus throughout the study; R5 switch, R5 isolates preceding the acquisition of CXCR4-using virus in patients with a detected switch to X4, R5X4, or multitropic virus; CXCR4 using, X4, R5X4, or multitropic isolates from the patients in the R5 virus switch group.
[b] p24 only, p24 antigen production but no syncytium formation.
[c] p24 + sync, syncytium induction and p24 antigen production.
[d] Significantly lower numbers and percentages of replicating R5 isolates were obtained from nonswitch virus patients than from R5 switch virus patients when two isolates from each patient were analyzed as described for Table 2 ($P = 0.01$, chi-square analysis).

TABLE 2. Changes within the R5 phenotype over time

Patient[a]	Patient code[b]	No. of R5 isolates	Time between first and last R5 isolate (mo)	CD4 cell count (10^6 cells/liter) at the time of isolation		Phenotype of R5 isolate[c]		Antiretroviral therapy at time of isolation[e]	
				First	Last	First	Last	First	Last
R5 nonswitch									
292	I	2	41	140	90	Narrow	Broad(2)	AZT	AZT + ddI
435		10	54	730	462	Narrow	Narrow		
451	J	2	31	220	20	Broad(1)	Broad(1)	AZT	ddI
1047		14	99	630[d]	360	Broad(1)	Broad(1)		
1276		9	80	320	40	Narrow	Broad(2)		ddI
1838		4	63	455	290	Narrow	Broad(1)		
1703	F	2	27	310	190	Broad(1)	Narrow	AZT	AZT
2010	G	2	35	260	5	Broad(2)	Broad(3)	AZT	ddI
2061	L	2	44	220	13	Broad(3)	Broad(3)		
2146	M	2	74	750	20	Broad(2)	Broad(3)		AZT
2216	H	2	33	290	6	Broad(2)	Broad(3)		
2245	N	3	64	310	280	Broad(2)	Broad(1)		
3408	O	3	77	270	140	Broad(1)	Broad(2)		HAART
4021	Q	3	58	240	220	Broad(1)	Broad(1)	AZT	HAART
4468	R	2	18	200	9	Broad(1)	Broad(3)		AZT
R5 switch									
958		5	26	300[d]	30	Narrow	Broad(2)	AZT	
965		11	41	310	360[d]	Narrow	Broad(2)		AZT
1023		4	36	500	500	Broad(2)	Broad(3)		
1679		23	26	230	81	Broad(2)	Broad(3)		AZT
1991		13	39	260	160	Broad(2)	Broad(3)		AZT
2112		6	42	340[d]	338	Broad(1)	Broad(3)		AZT
2239		2	20	730	410	Broad(1)	Broad(1)		
2242		18	49	260	119	Broad(2)	Broad(3)		AZT
2282		7	34	350	163	Broad(3)	Broad(2)		AZT
2289		10	48	490[d]	250	Broad(2)	Broad(3)		AZT
3382		3	38	150	160	Broad(2)	Broad(1)		

[a] R5 nonswitch, R5 phenotype was maintained throughout the study period; R5 switch, later acquisition of CXCR4-using virus.
[b] Patient code used by Jansson and coauthors (29).
[c] Narrow, virus using wild-type CCR5 but no chimeric receptor; Broad(1) to Broad(3), virus able to use one to three chimeric receptors in addition to CCR5.
[d] $CD4^+$-T-cell count was not available at the exact time point; the closest previous value is used.
[e] AZT, zidovudine; ddI, didanosine; HAART, highly active antiretroviral therapy.

were analyzed separately, the correlation between viral phenotype and CD4 count was statistically significant in the nonswitch virus patient group ($P = 0.008$, Kruskal-Wallis test). These results indicate that during the HIV-1 pathogenic process, the loss of $CD4^+$ T cells parallels the evolution of the R5 phenotype within nonswitch virus patients.

While the evolution of receptor use from the $R5^{narrow}$ to the $R5^{broad}$ phenotype was significantly associated with CD4 decline, evolution in single patients appeared to be discontinuous and was associated with a striking fluctuation of virus variants able to use one (usually FC-2) or several chimeric receptors. These patterns of R5 virus evolution are illustrated for four patients, two of whom were infected with virus that maintained the R5 phenotype during the entire disease course and two who later acquired CXCR4-using isolates (Fig. 3).

The $R5^{narrow}$ phenotype as well as the different categories of the $R5^{broad}$ phenotype occurred in both the nonswitch and switch virus groups. However, differences in frequencies of the various phenotypes in the two groups were observed (Fig. 4). The majority of viruses (63%) in the R5 nonswitch group had the $R5^{narrow}$ or $R5^{broad(1)}$ phenotype, and conversely, as many as 80% of the viruses with the $R5^{broad(2)}$ and $R5^{broad(3)}$ phenotypes occurred in the R5 switch group (OR = 6.0; 95% CI,

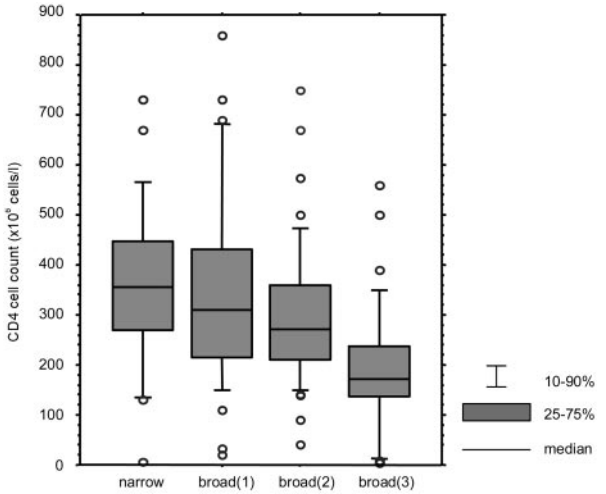

FIG. 2. Correlation between different R5 phenotypes, i.e., the $R5^{narrow}$, $R5^{broad(1)}$, $R5^{broad(2)}$, and $R5^{broad(3)}$ phenotypes, and CD4-T-cell counts. $CD4^+$-T-cell counts in the four different groups are significantly different when two isolates are analyzed from each patient as in Table 2 ($P = 0.04$, Kruskal-Wallis test). Determination of coreceptor use was based on p24 antigen production. We used R5 isolates from both nonswitch and switch virus patients for whom $CD4^+$ T cells were counted on the same day as virus isolation (a total of 142 isolates).

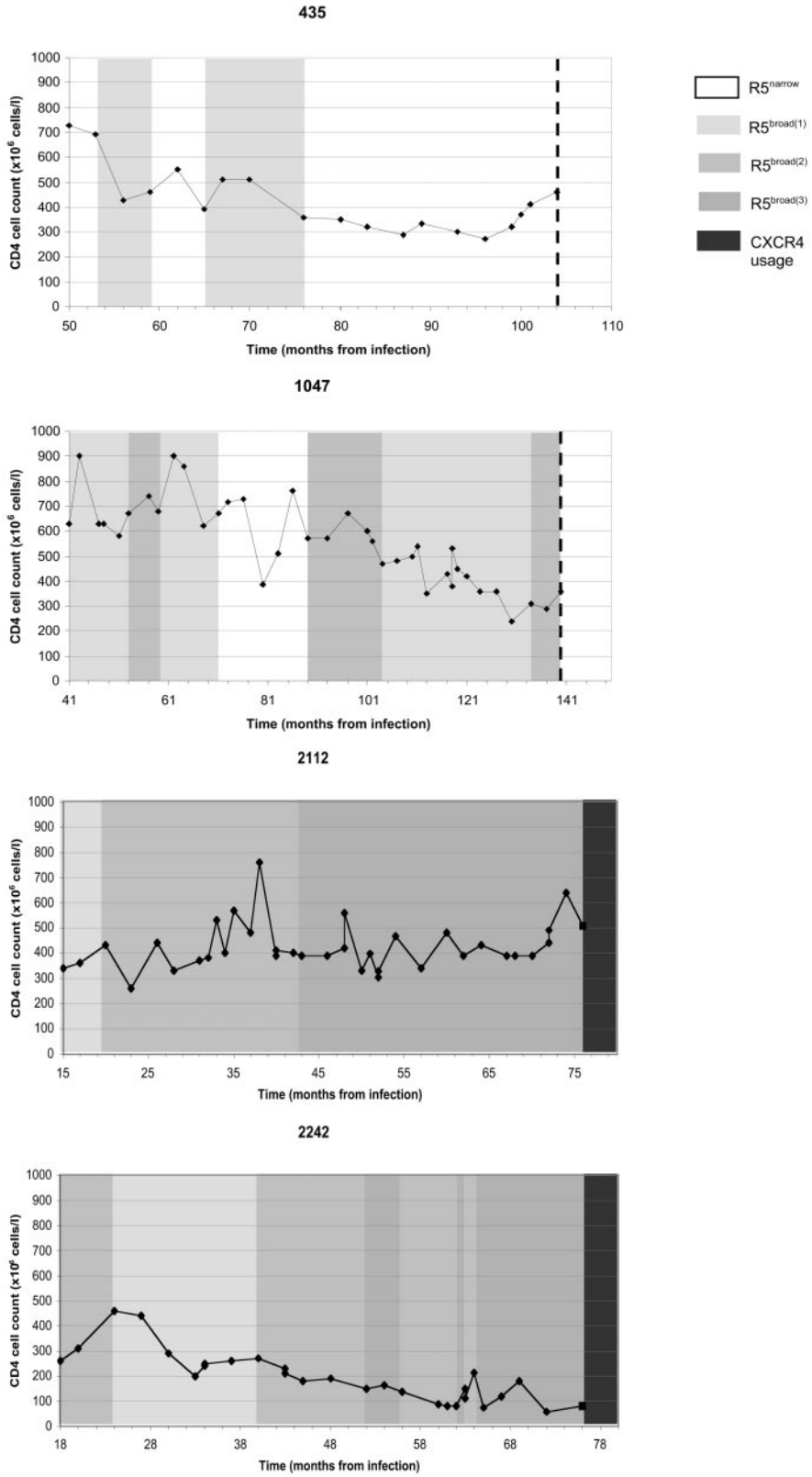

FIG. 3. $CD4^+$-T-cell counts and use of chimeric receptors of R5 viruses for four patients. Patients 435 and 1047 are nonswitch virus patients and yielded virus with the R5 phenotype throughout the study (10 and 14 isolates tested, respectively). The dashed line denotes the end of the study. Patients 2112 and 2242 are switch virus patients who later acquired CXCR4-using virus. Each virus was tested twice. The left axis and line show the $CD4^+$-T-cell counts as numbers of cells (10^6) per liter of blood. The four different shades of grey and white display changes in viral phenotypes over time, with white indicating the $R5^{narrow}$ phenotype; increasing darkness indicating the $R5^{broad(1)}$, $R5^{broad(2)}$, and $R5^{broad(3)}$ phenotypes; and the darkest grey indicating CXCR4-using virus. The infection date was calculated as the midpoint between the last negative and the first positive sample.

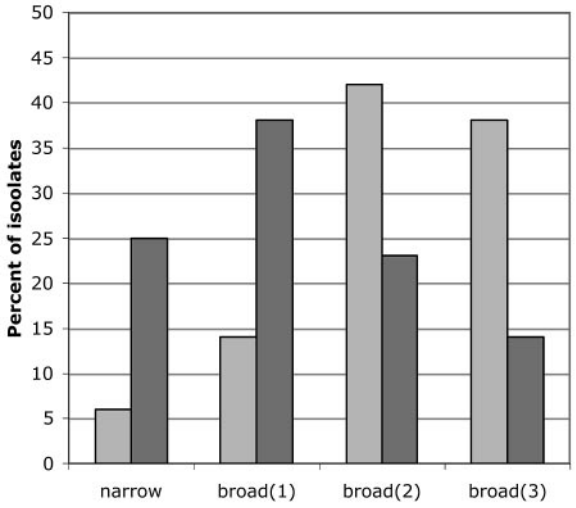

FIG. 4. Distribution of R5 isolates from nonswitch virus patients (dark gray, 64 isolates) and switch virus patients (light gray, 109 isolates) in the different R5 phenotypes; levels of viruses with the $R5^{narrow}$, $R5^{broad(1)}$, $R5^{broad(2)}$, and $R5^{broad(3)}$ phenotypes are shown as percentages of replicating isolates. The majority of viruses in the nonswitch virus group had the $R5^{narrow}$ or $R5^{broad(1)}$ phenotype, and conversely, most of the isolates in the switch virus group had the $R5^{broad(2)}$ or $R5^{broad(3)}$ phenotype. Statistical analysis was carried out with two isolates from each patient (compare Table 2) after adjustment for CD4 status (logistic regression) (OR = 6.0; 95% CI, 1.5 to 23.7; $P = 0.01$).

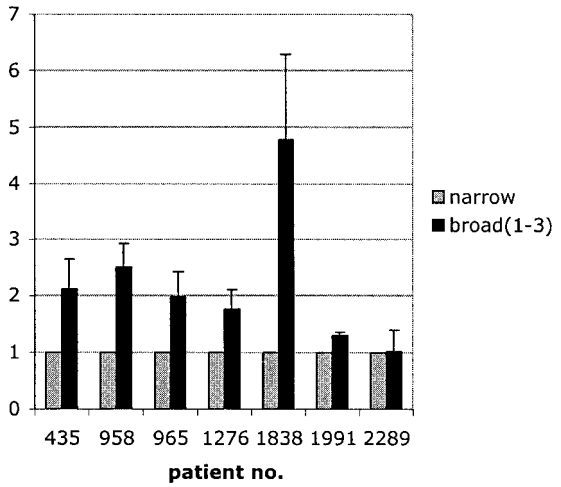

FIG. 5. Comparison of the infectivities of sequential $R5^{narrow}$ and $R5^{broad(1)}$ to $R5^{broad(3)}$ isolates from seven patients. A plaque assay with U87.CD4-CCR5 cells was used, and titers were determined at the same time with the same amount of p24 antigen per volume of virus supernatant. A pairwise comparison of sequential isolates with different R5 phenotypes was performed. Results are shown as ratios of numbers of plaque-forming units of $R5^{broad(1)}$, $R5^{(broad2)}$, and $R5^{(broad3)}$ isolates to $R5^{narrow}$ isolates per milliliter, where the infectivity of $R5^{narrow}$ was set at 1. Error bars show intra-assay variation. Infectious titers of $R5^{broad(1)}$, $R5^{(broad2)}$, and $R5^{(broad3)}$ isolates were significantly higher than those of isolates with the $R5^{narrow}$ phenotype ($P = 0.04$, Wilcoxon signed-rank test).

1.5 to 23.7; $P = 0.01$) when the R5 switch group was compared with the R5 nonswitch group and adjustment was made for CD4 status (logistic regression), using two isolates from each patient as described in Table 2. This finding suggests that while evolution of the R5 phenotype appears to be a general phenomenon, evolution is more pronounced in patients whose virus later switches to CXCR4 use.

Considering the use of FC-4b only, R5 viruses from switching individuals may more frequently utilize this chimeric receptor than R5 viruses from those not switching ($P = 0.01$, chi-square test) (Table 1). This suggests that R5 switch isolates are more flexible in their use of the CCR5 receptor than R5 nonswitch isolates since the second and third extracellular loops together are sufficient for infection to take place.

Infectivities of R5 viruses with different phenotypes at the wild-type CCR5 receptor. In order to clarify whether the ability to use a broad range of chimeric receptors also influences infectivity, we compared the infectious titers of viruses with the $R5^{narrow}$ and $R5^{broad}$ phenotypes in a plaque assay using U87.CD4-CCR5 cells (54). A pairwise comparison of infectious titers of sequential R5 viruses with different phenotypic characteristics, derived from individual patients, was performed. A titer comparison, illustrated in Fig. 5 with ratios of $R5^{broad}$ to $R5^{narrow}$ viruses isolated sequentially from seven individuals, showed that $R5^{broad}$ isolates had significantly higher infectious titers than $R5^{narrow}$ isolates ($P = 0.04$, Wilcoxon signed-rank test). Thus, viruses with a broad capacity to use CCR5 and CXCR4 chimeric receptors also showed increased infectivity on CCR5-expressing cells.

Resistance to inhibition by RANTES is related to chimeric receptor use. In an earlier work (29) 24 isolates from 12 patients in the R5 nonswitch virus group were tested on PBMC for sensitivity to inhibition by the CC chemokine RANTES. The results showed that R5 viruses with reduced RANTES sensitivity could be isolated from five patients with severe immune suppression. With this in mind, we asked if there is any relationship between RANTES sensitivity and chimeric receptor usage. We plotted RANTES IC_{50} data previously obtained (29), together with the results of RANTES inhibitions performed with 11 isolates from an additional four patients in the R5 nonswitch virus group (a total of 35 R5 isolates from 16 patients) against the use of chimeric receptors (Fig. 6). This analysis revealed a significant correlation between the evolution of the R5 phenotype and sensitivity to inhibition by RANTES ($P = 0.01$, Kruskal-Wallis test). Viruses with the $R5^{narrow}$ phenotype had the lowest RANTES IC_{50}s, while $R5^{broad(3)}$ viruses had the highest (Fig. 6). Thus, the mode of the R5 virus-coreceptor interaction, monitored through chimeric receptor use, translated into RANTES inhibition sensitivity. Further analysis also showed a significant inverse correlation ($P = 0.04$ according to Spearman rank correlation analysis) between CD4-T-cell counts at the time of R5 virus isolation and the RANTES sensitivity of corresponding isolates (data not shown). Our observations suggest that during HIV-1 disease progression, an increased resistance to inhibition by RANTES is concomitant with the evolution of the R5 phenotype.

DISCUSSION

The use of receptor chimeras between CXCR4 and CCR5 allowed us to gain further insight into HIV biological variation. We found that HIV-1 isolates able to use CCR5 but not CXCR4 (R5 phenotype) vary in their capacity to use chimeric

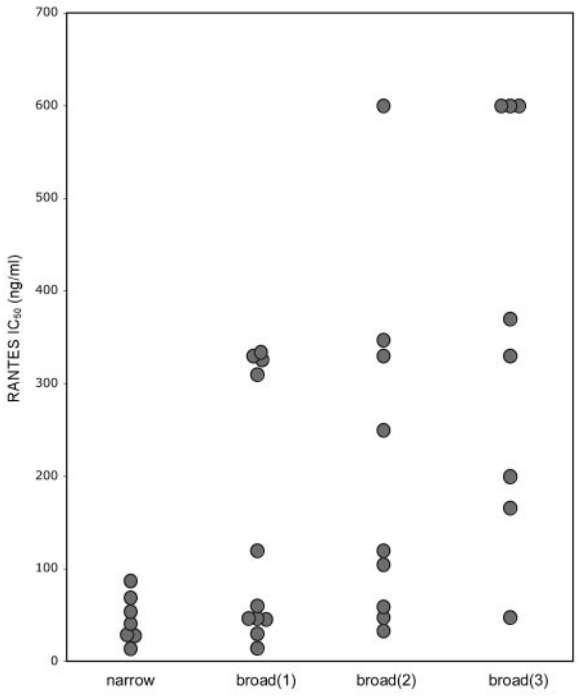

FIG. 6. Correlation between chimeric receptor use and RANTES sensitivity of 35 R5 isolates from patients maintaining the R5 phenotype during the entire disease course. A broader use of chimeric CCR5 and CXCR4 receptors correlated with reduced RANTES sensitivity ($P = 0.01$, Kruskal-Wallis test). RANTES sensitivity is indicated with IC_{50}s.

receptors. The change from an exclusive use of wild-type CCR5 ($R5^{narrow}$) to the use of increasing numbers of chimeric receptors [$R5^{broad(1)}$ to $R5^{(broad3)}$] over time is interpreted as the evolution to extended flexibility in the use of the CCR5 receptor. Viruses with flexible CCR5 use had higher infectivity at the wild-type CCR5 than isolates with the $R5^{narrow}$ phenotype. Moreover, a broadened R5 phenotype also correlated with a reduced number of $CD4^+$ T cells within nonswitch virus patients, indicating that the increased flexibility of R5 isolates to use CCR5 seen in vitro is also relevant in vivo and may influence disease progression.

Our results show that the evolution of HIV-1 coreceptor use in the course of disease progression not only encompasses a switch to CXCR4 use but is a process that involves the evolution of the R5 phenotype as well. $R5^{narrow}$ isolates were preferentially isolated from patients with the highest $CD4^+$-T-cell counts, while $R5^{broad(3)}$ isolates were most frequently found during severe immunodeficiency. Viruses evolved to a more flexible and more efficient CCR5 use even if a switch to CXCR4 use occurred later during the observation period. However, the broader R5 phenotypes were more common in patients whose viruses switched phenotypes than in patients who retained R5 phenotype virus throughout the course of disease.

Previous studies using mutagenesis and biochemistry have suggested that CCR5 binding and host cell infection of R5 HIV-1 variants depend on the highly acidic and tyrosine-rich amino-terminal region of CCR5 (20, 23). However, other regions of CCR5, such as the second extracellular loop, have also been implied to play a role in infection by R5 viruses (38, 44). Similar observations were noted in studies using various types of receptors (1, 9, 17, 46, 49). In a recent study by Platt et al., the passage of R5 virus on cells expressing CCR5 containing amino-terminal mutations resulted in adapted viruses with mutations in the gp120 V3 loop (47). Adaptation led to enhanced fusogenicity and was interpreted by those authors such that the viruses either were not dependent on the affinity between gp120 and CCR5 or showed enhanced affinity to the coreceptor. Interestingly, the apparent increased affinity of gp120 to CCR5 depended on interaction with the second extracellular loop and not the N terminus. It is likely also in our study that the interaction of R5 viruses with receptor chimeras occurs through the second extracellular loop and that this confers increased receptor affinity and the capacity to yield higher infectious titers at wild-type CCR5. Indeed, it was previously reported that gp120s of different R5 viruses may bind CCR5 with different affinities (16, 64). In the present study, we noted a striking difference in the use of one chimeric receptor, FC-4b, by R5 isolates derived from patients later yielding CXCR4-using isolates and R5 isolates from those patients continuing to yield only R5 isolates. Thus, these results suggest that R5 variants with the ability to use FC-4b contain envelope structures that are more prone to mutate towards CXCR4 usage.

What is the selective pressure that drives HIV-1 to a flexible and more efficient use of CCR5? Our results show that resistance to inhibition by the CCR5 ligand RANTES parallels the evolution of CCR5 coreceptor use by the virus and disease progression in the patient. We suggest that HIV-1 variants that develop RANTES resistance may have an advantage and successfully compete for the receptor even in the presence of the natural ligand(s). It is possible that the selection is driven by RANTES or any other CCR5 ligand(s). We previously reported on the evolution of R5 isolates in relation to reduced RANTES sensitivity at the time of severe immunosuppression within patients maintaining CCR5-dependent isolates during the entire disease course (29, 30). This observation has recently also been confirmed by Koning and coauthors (34). Thus, in relation to chimeric receptor use, it is intriguing to note that among isolates from patients infected with a virus that maintains the R5 phenotype, we found that broadness in chimeric receptor use correlated with resistance to RANTES inhibition. Taken together, these observations suggest that virus-coreceptor interactions may be altered also within infected individuals who develop AIDS while continuing to yield truly CCR5-dependent isolates. Experimentally, it has been shown that in vitro passage of an R5 virus in the presence of a CCR5 antagonist selected for a highly resistant escape mutant still dependent on CCR5 for host cell entry (58). Variation in affinity has also been shown to translate into divergence in R5 virus sensitivity to entry inhibitors such as T20 and TAK-779 (48). Thus, our findings of R5 variants with RANTES resistance and broadened flexibility during coreceptor binding, emerging concomitantly with disease progression, may be important in the optimal design of new, effective HIV-1 entry inhibitors.

An alternative mechanism for the selection of R5 variants with an altered receptor interaction may be a reduced expression of CCR5 on target cells. Down-regulation of CCR5 as a consequence of CCR5-ligand binding or altered cytokine bal-

ance has been suggested to occur during HIV-1 disease progression (3, 59). Several cytokines, such as IL-4, IL-7, and IL-10, have been shown to down-regulate CCR5, and increased expression of these cytokines has also been implied in the triggering of the HIV-1 coreceptor switch (40, 45, 60). The selective loss of certain $CD4^+$-cell subsets during HIV-1 disease progression has also been implied to occur in the selection of certain virus variants (5, 61). In line with this, we believe that R5 variants with an altered CCR5 engagement also may emerge as a consequence of changed CCR5 availability.

In summary, we show that HIV-1 evolves in vivo towards a more efficient use of CCR5. It is likely that this is a selection process that favors the replication of virus variants that bind CCR5 in an alternative way, or with higher affinity, as a consequence of competition with the natural ligand or changed CCR5 availability.

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