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The CCR5 and CXCR4 Coreceptors Are Both Used by Human Immunodeficiency Virus Type 1 Primary Isolates from Subtype C

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Human immunodeficiency virus type 1 (HIV-1) subtype C viruses with different coreceptor usage profiles were isolated from 29 South African patients with advanced AIDS. All 24 R5 isolates were inhibited by the CCR5-specific agents, PRO 140 and RANTES, while the two X4 viruses and the three R5X4 viruses were sensitive to the CXCR4-specific inhibitor, AMD3100. The five X4 or R5X4 viruses were all able to replicate in peripheral blood mononuclear cells that did not express CCR5. When tested using coreceptor-transfected cell lines, one R5 virus was also able to use CXCR6, and another R5X4 virus could use CCR3, BOB/GPR15, and CXCR6. The R5X4 and X4 viruses contained more-diverse V3 loop sequences, with a higher overall positive charge, than the R5 viruses. Hence, some HIV-1 subtype C viruses are able to use CCR5, CXCR4, or both CXCR4 and CCR5 for entry, and they are sensitive to specific inhibitors of entry via these coreceptors. These observations are relevant to understanding the rapid spread of HIV-1 subtype C in the developing world and to the design of intervention and treatment strategies.

The envelope glycoproteins of human immunodeficiency virus type 1 (HIV-1) mediate viral entry into cells. This process requires the sequential interaction of gp120 with two host surface proteins, namely CD4 and a chemokine receptor, most often CCR5 or CXCR4 (15). Transmission of HIV-1 is almost always associated with viruses that utilize CCR5 (R5 viruses), which predominate during the acute and asymptomatic phases of infection (31, 40). Disease progression is often associated with the emergence of viruses that have acquired the ability to use CXCR4 (X4 viruses) instead of, or as well as, CCR5 (R5X4 viruses) (10, 42). These patterns of coreceptor usage correspond to the phenotypes previously defined by the MT-2 assay; here, syncytium-inducing (SI) viruses use CXCR4, while non-syncytium-inducing (NSI) viruses use CCR5 (3). Some isolates from AIDS patients can also use other chemokine receptors as coreceptors in receptor-transfected cell lines, including CCR1, CCR2b, CCR3, BOB (GPR15), and CXCR6 (Bonzo/STRL33) (14, 18, 39, 45, 61, 63; G. Alkhatib, F. Liao, E. A. Berger, J. M. Farber, and K. W. Peden, Letter, Nature **388:**238, 1997). Rarely, however, are such alternative coreceptors used in primary cells (45, 64).

Agents that target CCR5 or CXCR4 can block HIV-1 entry and prevent infection in vitro. These include RANTES, a natural chemokine ligand for CCR5; PRO 140, a mouse monoclonal antibody directed at CCR5; TAK-779, a small molecule that binds to a pocket within CCR5 transmembrane helices 1, 2, 3, and 7; and SCH-C, another small-molecule, CCR5 antagonist (2, 17, 36, 47, 53, 60; J. Reynes, Abstr. 2nd Collaborative Res. Semin. HIV Other Viral Entry Inhibitors, abstr., 2002). Among these, SCH-C is currently in clinical trials and has been found to have antiviral activity (J. Reynes, R. Rouzier, T. Kanouni, V. Baillat, B. Baroudy, A. Keung, C. Hogan, M. Markowitz, and M. Laughlin, Abstr. 9th Conf. Retrovir. Opportun. Infect., abstr. 1, 2002.). AMD3100 is a small-molecule CXCR4 antagonist with activity against X4 viruses in vitro and antiviral activity in vivo, although it is no longer being pursued clinically because of pharmacology and toxicology considerations (16, 25, 43; G. Bridger, Abstr. 2nd Collaborative Res. Semin. HIV Other Viral Entry Inhibitors, abstr., 2002; C. Hendrix, A. C. Collier, M. Lederman, R. Pollard, S. Brown, M. Glesby, C. Flexner, G. Bridger, K. Badel, R. MacFarland, G. Henson, G. Calandra, et al., Abstr. 9th Conf. Retrovir. Opportun. Infect., abstr. 391-T, 2002.). Collectively these molecules are members of a new class of antiretroviral drugs termed entry inhibitors, some of which are being tested for their abilities to prevent or treat HIV-1 infection (29, 33, 44).

HIV-1 subtype C now accounts for more than half of new infections worldwide and is now the most prevalent subtype (www.unaids.org). It is associated with the rapidly expanding epidemics of southern Africa, India, and China. Previous studies have reported that subtype C isolates almost exclusively use the CCR5 coreceptor, with CXCR4 usage being only very rarely observed. This includes studies of isolates from India, Ethiopia, Malawi, and South Africa (1, 4, 7, 34, 38). HIV-1 cellular tropism and coreceptor specificity are largely determined by the sequence of the third hypervariable loop (V3) of the viral gp120 glycoprotein, and distinct changes in this region have been associated with the NSI/SI phenotype among subtype B viruses (8, 12, 28, 46). For example, the presence of a neutral amino acid (S) at position 11 and a negatively charged amino acid (D or E) at position 25 correlates with the NSI

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phenotype. Conversely, a basic residue at one of these positions, which increases the overall positive charge of the V3 loop, can change the viral phenotype from NSI to SI (11, 26, 32). Compared to other subtypes, the V3 regions of the subtype C viruses studied to date tend to be highly conserved and have a low overall positive charge, which is consistent with the NSI phenotypes and restricted coreceptor usage of subtype C viruses (23).

The above observations have created the impression that subtype C viruses might have envelope glycoproteins that are atypical in structure or function compared to viruses from other HIV-1 subtypes. If this were truly the case, intervention strategies, including the design of vaccines based on neutralizing-antibody induction, as well as therapeutic approaches based on inhibiting viral entry, might need to be designed differently for use in areas where subtype C viruses predominate. Moreover, an atypical property of the subtype C envelope glycoproteins might, in principle, help to explain why subtype C viruses are now spreading so rapidly in southern Africa and elsewhere, as has been proposed (5, 20, 35). However, an important caveat to the early studies on the coreceptor usage of subtype C strains is that they focused predominantly on isolates from patients who were in the relatively early stages of disease or whose clinical status was poorly defined (1, 4, 7, 34, 38, 51). Yet in studies on North American or European cohorts, the acquisition of CXCR4 use by subtype B viruses increases with time after infection and as the disease becomes more severe. This raises the possibility that the predominant usage of CCR5 by subtype C isolates was due more to a sampling artifact than to any fundamental, biological property of these viruses. We have therefore isolated viruses from patients with subtype C infection and advanced AIDS to see whether SI viruses are identified more frequently under these conditions than hitherto. We have also explored whether subtype C isolates able to use either or both of CCR5 and CXCR4 are sensitive to coreceptor-specific entry inhibitors.

Isolation of NSI and SI HIV-1 subtype C viruses. Patients admitted to the Sizwe Infectious Diseases Hospital in Johannesburg, South Africa, between August and December 1999 with HIV-1-related opportunistic infections and CD4-T-cell counts of less than 100 cells/μl were selected for this study. Ethical clearance was obtained from the University of the Witwatersrand Committee for Research on Human Subjects (Medical), and informed consent was obtained from all patients prior to drawing 5 ml of blood in EDTA. The study cohort comprised 13 men and 16 women with a median age of 34 years and a median CD4 count of 40 cells/μl (Table 1). Levels of virus in plasma were measured using the Versant HIV-1 RNA 3.0 assay (bDNA assay from Bayer Nucleic Acid Diagnostics), previously shown to amplify HIV-1 RNA from all HIV-1 group M genetic subtypes (19, 50). The median viral load in plasma for this cohort was high, at 321,070 HIV-1 RNA copies/ml, consistent with advanced HIV-1 disease (Table 1). Twenty-two patients had tuberculosis (TB), four had cryptococcal meningitis (CM), two had *Pneumocystis carinii* pneumonia (PCP), and one had mycobacteria other than tuberculosis. HIV-1 was isolated from 22 patients when their peripheral blood mononuclear cells (PBMC) were cocultured with phytohemagglutinin (PHA)-activated donor PBMC as previously described (34). Isolates from the remaining seven patients were obtained by culturing 100 μl of plasma overnight with PHA-activated PBMC. Culture supernatants were tested weekly using a commercial p24 antigen kit (Beckman Coulter Ltd., Buckinghamshire, United Kingdom) and expanded when high levels of p24 antigen were present. Cultures were replenished with fresh interleukin 2 (IL-2) medium every 3 to 4 days and with PHA-stimulated donor PBMC every 7 days for up to 3 weeks. Virus-containing supernatants were spun to remove cellular debris and frozen at −70°C until use. Some isolates were reexpanded using PBMC stimulated with both PHA and surface-immobilized anti-CD3 monoclonal antibody plus IL-2 as previously described (54) to generate high titer stocks for further experiments. Isolates therefore had limited expansion in PBMC in vitro and are likely to reflect the viral swarm present in vivo, although we cannot exclude that some selection, albeit limited, occurred during in vitro passage.

TABLE 1. Characteristics of 29 South African AIDS patients with NSI or SI isolates

Parameter	Value for group[a]		
	Total cohort	NSI isolates	SI isolates
No. studied (% of total)	29	24 (83)	5 (17)
No. female	16	18	3
No. male	13	11	2
Median age	34	35	29
Median viral load	321,070	776,090*	68,410*
Median CD4 count	40	48**	10**
Diagnosis			
Tuberculosis	22	19	3
Cryptococcal meningitis	4	2	2
PCP	2	2	0
MOTT/Mac	1	1	0

[a] *, $P < 0.05$ by Mann-Whitney test. **, $P < 0.05$ by Mann-Whitney test.

Viruses were tested for their ability to cause syncytia in the MT-2 T-cell line (34). Among the 29 isolates, 5 (17%) were able to grow in MT-2 cells, leading to syncytium formation (SI isolates) (Table 1). The subset of patients with SI viruses had significantly reduced CD4-T-cell counts, which is similar to observations made with patients infected with subtype B strains (41). Viral loads among the patients with SI viruses were not particularly high compared to levels in the cohort as a whole, perhaps because too few memory CD4 T cells remained to support high-level viral replication (Table 1). However, viral load measurements were available from only three patients with SI viruses, so no conclusions can be drawn from the statistical perspective. SI viruses were isolated from 3 of 22 (14%) TB patients and 2 of 4 (50%) CM patients. However, overall there were no significant differences in the age, gender, or opportunistic infections of patients with SI isolates compared to those that harbored NSI isolates. A cohort containing larger numbers of patients with SI isolates will be needed to fully address such issues.

The HIV-1 isolates were subtyped in the C2-V5 region of the *env* gene by a heteroduplex mobility assay (HMA), and all were shown to belong to HIV-1 subtype C, the predominant subtype in South Africa (6, 58). Thirteen isolates were also subtyped in *gag* by HMA and shown to belong to subtype C (data not shown), suggesting that these isolates were unlikely to be intersubtype recombinants, although this cannot be ex-

TABLE 2. Coreceptor usage by HIV-1 subtype C isolates from AIDS patients

Isolate	Source	CD4 count (cells/μl)	Viral load (RNA copies/ml)	Subtype of *env* gene	MT-2 assay result	p24 antigen level[b]					Biotype
						U87.CD4 cells expressing:		GHOST.3 cells expressing:			
						CXCR4	CCR5	CXCR6	CCR3	BOB	
PCP1	PBMC	2	NA[a]	C	NSI	−	++++	+++	−	−	R5
CM1	PBMC	43	146,514	C	NSI	−	++	−	−	−	R5
CM7	PBMC	79	14,089	C	NSI	−	++++	−	−	−	R5
SW2	Plasma	84	157,150	C	NSI	−	++++	−	−	−	R5
SW3	Plasma	53	261,880	C	NSI	−	++++	−	−	−	R5
SW4	Plasma	76	1,496,620	C	NSI	−	++++	−	−	−	R5
SW5	PBMC	40	1,374,235	C	NSI	−	++++	−	−	−	R5
SW8	Plasma	67	1,198,880	C	NSI	−	+	−	−	−	R5
SW9	Plasma	65	301,605	C	NSI	−	++++	−	−	−	R5
SW14	PBMC	32	5,000,000	C	NSI	−	+++	−	−	−	R5
SW16	PBMC	9	151,630	C	NSI	−	+	−	−	−	R5
SW22	PBMC	67	2,749,340	C	NSI	−	++	−	−	−	R5
SW23	PBMC	10	696,000	C	NSI	−	++++	−	−	−	R5
SW26	PBMC	25	NA	C	NSI	−	++++	−	−	−	R5
SW28	Plasma	43	3,814,130	C	NSI	−	++++	−	−	−	R5
SW29	PBMC	12	1,190,180	C	NSI	−	++++	−	−	−	R5
SW34	PBMC	40	127,000	C	NSI	−	++++	−	−	−	R5
SW35	PBMC	37	1,652,870	C	NSI	−	++++	−	−	−	R5
SW36	PBMC	55	201,340	C	NSI	−	++++	−	−	−	R5
SW37	PBMC	121	34,210	C	NSI	−	+	−	−	−	R5
SW38	PBMC	37	1,777,930	C	NSI	−	++++	−	−	−	R5
SW39	PBMC	94	321,070	C	NSI	−	++++	−	−	−	R5
SW40	PBMC	67	856,180	C	NSI	−	++++	−	−	−	R5
SW41	PBMC	60	3,162,240	C	NSI	−	+++	−	−	−	R5
CM9	PBMC	24	NA	C	SI	++++	++++	+++	+++	+++	R5X4
SW7	PBMC	10	NA	C	SI	++++	−	−	−	−	X4
SW12	Plasma	27	68,410	C	SI	++++	−	−	−	−	X4
SW20	PBMC	2	43,595	C	SI	++	++++	−	−	−	R5X4
SW30	PBMC	2	73,860	C	SI	++++	++++	−	−	−	R5X4

[a] NA, not available.
[b] p24 antigen level on day 12 (endpoint p24 assay). −, <250 pg of p24 antigen/ml; +, 250 pg to 1 ng of p24 antigen/ml; ++, 1 to 2 ng of p24 antigen/ml; +++, 2 to 4 ng of p24 antigen/ml; ++++, >4 ng of p24 antigen/ml.

cluded. Two viruses, CM9 and SW7, have been confirmed as being subtype C throughout its genome (37).

Coreceptor usage of HIV-1 subtype C isolates in transfected cell lines. Viral isolates were tested for their abilities to replicate in U87.CD4 cells transfected with either CCR5 or CXCR4, as previously described (34). Cells were plated in 12-well plates at 10^5 cells/well in 2 ml of selection medium (Dulbecco's modified Eagle's medium containing 10% fetal calf serum, antibiotics plus 500 μg of G418 (Boehringer Mannheim GmbH)/ml, and 1 μg of puromycin (ICN Biomedicals Inc., Aurora, Ohio)/ml. The following day, 1,000 50% tissue culture infectious doses were added. After incubation overnight, the cultures were washed three times with Dulbecco's modified eagle's medium plus 10% fetal calf serum to remove unbound virus and then monitored for syncytium formation and p24 antigen production on days 4, 8, and 12. The in-house p24 antigen enzyme-linked immunosorbent assay has a detection limit of 250 pg/ml (52). Isolates that induced syncytium formation and generated increasing concentrations of p24 antigen were considered to be replication positive.

There was an absolute correlation between the NSI and SI phenotypes and the ability of an isolate to use CCR5 or CXCR4, respectively. Thus, all 24 NSI isolates were able to replicate in CCR5-expressing cells but not in the CXCR4-expressing cells (hence, they are conventionally designated R5 viruses) (Table 2). The five SI isolates all replicated in CXCR4-expressing cells. Among them, three could replicate in CCR5-expressing cells (designated R5X4 viruses) while two did not (designated X4 viruses). Although PCP1 was classified as an R5 virus, it did show low-level usage of CXCR4 that was below the cutoff for the p24 antigen assay (data not shown). The ability of this virus to use CXCR6 (see below) very efficiently may account for these low levels of replication, since U87.CD4 cells express this receptor, and also GPR1, endogenously (21, 56).

In order to explore whether viral isolates were also able to use alternate HIV-1 coreceptors, U87.CD4 cell lines expressing CCR1, CCR2b, or CCR3 and GHOST.3 cell lines expressing BOB/GPR15 or CXCR6 were used. The GHOST.3 cell assays were performed essentially as described for the U87.CD4 cell assays except that cells were grown in the presence of 100 μg of hygromycin (Sigma-Aldrich, Johannesburg, South Africa)/ml. Because GHOST.3 cell lines express endogenous CXCR4, it was necessary to add 1.3 μM AMD3100 to prevent entry via this coreceptor (62, 64). Viral replication in the presence of AMD3100 therefore indicated use of the transfected coreceptor (either BOB/GPR15, CCR3, or CXCR6). Two viruses were able to use alternate coreceptors efficiently. Thus, PCP1 could use CXCR6, while CM9 could use CXCR6, BOB/GPR15, and CCR3 (Table 2). In each case, entry via the

TABLE 3. Inhibition of HIV-1 subtype C viruses by CCR5 and CXCR4 inhibitors

Virus	Subtype	Phenotype	% Inhibition by CCR5 inhibitor[a]				% Inhibition by CXCR4 inhibitor AMD3100 at[a]:	
			RANTES at:		PRO 140 at:			
			19 nM	4 nM	167 nM	33 nM	500 nM	100 nM
PCP1	C	R5, CXCR6	**97**	*68*	**98**	*88*	*34*	*18*
CM1	C	R5	*87*	*49*	*78*	*59*	*0*	*8*
CM7	C	R5	**94**	*89*	**97**	**100**	*3*	*19*
SW5	C	R5	**100**	*75*	**100**	**100**	*19*	*6*
SW23	C	R5	**100**	*89*	**98**	**94**	*0*	*0*
SW26	C	R5	**100**	**91**	**93**	*87*	*8*	*0*
SW29	C	R5	**98**	*74*	**98**	**91**	*13*	*0*
SW38	C	R5	**100**	*34*	**100**	**96**	*0*	*0*
CM9	C	R5X4, CXCR6, BOB, R3	*36*	*36*	*34*	*13*	**97**	*58*
SW7	C	X4	*0*	*24*	*0*	*5*	**100**	*87*
SW12	C	X4	*0*	*0*	*0*	*0*	*86*	*31*
SW20	C	R5X4	*0*	*0*	*79*	*0*	**100**	*0*
SW30	C	R5X4	*24*	*9*	*8*	*39*	*85*	*3*
DJ258	A	R5	**100**	*82*	**100**	**98**	*46*	*0*
92RW026	A	R5	**98**	*69*	**100**	**100**	*63*	*23*
JR-FL	B	R5	**99**	*71*	**100**	**98**	*0*	*36*
92US714	B	R5	**95**	*60*	**100**	**100**	*0*	*0*
92US077	B	R5X4	*17*	*48*	*48*	*41*	**99**	*76*
CM235	E	R5	**95**	*62*	**100**	**97**	*22*	*2*
92TH001	E	R5	**100**	*88*	**99**	**98**	*60*	*21*

[a] Inhibition of >90% is bolded, and inhibition of <90% is in italics.

alternate coreceptor was at a level of efficiency comparable to that mediated by CCR5 or CXCR4, in that the extent of virus production was similar in the different cell cultures (Table 2).

Sensitivity of subtype C isolates to CCR5 and CXCR4 inhibitors in PBMC. To confirm the use of CCR5 or CXCR4 in PBMC, viral isolates were cultured in the presence of CCR5-specific (RANTES and PRO 140) or CXCR4-specific (AMD3100) entry inhibitors. A subset of 13 subtype C viruses able to use different coreceptors was selected for analysis, in comparison with a panel of 7 isolates from subtypes A, B, and E (from National Institutes of Health Reference and Reagent Repository). PBMC used in these experiments were depleted of $CD8^+$ T cells using a RosetteSep CD8 antibody cocktail (StemCell Technologies, Vancouver, Canada). The reduction in p24 levels was calculated relative to cultures without inhibitors, and the percentage of inhibition at a high and low concentration of each inhibitor is reported. Results shown are from duplicate cultures, and each experiment was repeated twice as previously described (52).

The replication of seven of the eight R5 subtype C viruses was effectively inhibited (>90%) by the highest concentrations of RANTES and PRO 140 that were tested (Table 3). One such isolate (CM1) was marginally less sensitive to RANTES (87% inhibition) and PRO 140 (78% inhibition). In contrast, the CCR5 inhibitors were ineffective against R5X4 and X4 viruses, suggesting that these viruses can all use CXCR4 to enter PBMC efficiently. AMD3100 effectively inhibited all the R5X4 and X4 viruses, although two isolates (SW12 and SW30) were slightly less sensitive (86 and 85% inhibition, respectively) than the others. The ability of AMD3100 to restrict the replication of all three R5X4 viruses suggests that they use CXCR4 in preference to CCR5 to enter PBMC in vitro. As expected, AMD3100 was ineffective against all the R5 isolates, because it is known not to interfere with CCR5-mediated entry (16, 43). The ability of an isolate to use alternate coreceptors in transfected cell lines did not influence whether it was sensitive to any of the entry inhibitors in PBMC cultures. Thus, both PCP1 and CM9 were fully sensitive to RANTES, PRO 140, and AMD3100 despite being able to use alternate coreceptors (Table 2). Either expression levels of the alternate coreceptors on PBMC are lower than on the transfected cell lines or these coreceptors simply do not function as HIV-1 entry receptors on primary cells (45).

The CCR5 inhibitors were also effective against the R5 isolates from subtypes A, B, and E. One R5X4 subtype B isolate (92US077) was effectively inhibited by AMD3100 but not by RANTES and PRO 140, suggesting that it too uses CXCR4 preferentially to enter PBMC. Overall, there was a good, and subtype-independent, correlation between the classification of an isolate as R5 or X4 by use of the coreceptor-transfected cell lines and its sensitivity to CCR5- or CXCR4-specific entry inhibitors in PBMC (52, 55). However, the isolates classified as R5X4, because they could use both CCR5 and CXCR4 in coreceptor-transfected cells (Table 2), were sensitive only to the CXCR4 inhibitor AMD3100 in PBMC and were little affected by the CCR5 antagonists PRO 140 and RANTES (Table 3). Hence, CXCR4 is the coreceptor of choice for these R5X4 isolates in primary T cells, with only a minority of the viruses present entering the cells via CCR5. The quantitative limitations of coreceptor-transfected cells for viral phenotyping have been previously discussed (62–64).

Replication of NSI and SI viruses in wild-type and CCR5 Δ32/Δ32 PBMC. The subtype C isolates used above were also tested for their ability to replicate in PBMC deficient in CCR5 (i.e., cells from an HIV-1-negative individual whom we confirmed by PCR to have the *CCR5* Δ*32*/Δ*32* genotype) (59).

TABLE 4. HIV-1 subtype C replication in PBMC from Δ32/Δ32 and wt/wt donors

Isolate	p24 antigen level for genotype[a]	
	Δ32/Δ32	wt/wt[b]
R5		
PCP1	−	++++
CM1	−	++++
CM7	−	++++
SW5	−	+++
SW23	−	++++
SW26	−	++++
SW29	−	++++
SW38	−	++++
R5X4		
CM9	+	++
SW20	+/−	+
SW30	+	++++
X4		
SW7	+	+++
SW12	++	+++

[a] p24 antigen level on day 8 (kinetic p24 assay). −, <250 pg of p24 antigen/ml; +, 250 pg to 10 ng of p24 antigen/ml; ++, 10 to 30 ng of p24 antigen/ml; +++, 30 to 60 ng of p24 antigen/ml; ++++, >60 ng/ml p24 antigen/ml.
[b] wt, wild type.

PBMC from the CCR5 Δ32/Δ32 donor and also from CCR5 +/+ control donors were isolated and stimulated using PHA and surface-immobilized anti-CD3 monoclonal antibody, as described above. On the following day, the cultures were washed three times and incubated in IL-2-containing medium. Supernatants were assayed for p24 antigen levels using a commercial assay with a kinetic readout (DuPont/NEN Life Sciences, Boston, Mass.) on days 4, 8, and 11.

None of the R5 isolates was able to replicate in the CCR5 Δ32/Δ32 PBMC, but all replicated well in the CCR5 +/+ PBMC, confirming their dependence on CCR5 to infect cells (Table 4). The R5X4 and X4 isolates were able to replicate in both the CCR5 Δ32/Δ32 and the CCR5 +/+ PBMC, although p24 production from the CCR5 Δ32/Δ32 cells was relatively low. In particular, the replication of isolate SW20 was sporadic in the CCR5 Δ32/Δ32 PBMC, with only some of the replicate wells producing p24 antigen. This contrasts with the consistent replication of the same virus in the CCR5 +/+ PBMC.

CXCR4-using subtype C viruses show alterations in V3 loop. The V3 loop is involved in determining coreceptor usage (27). Certain genetic changes in this region are associated with CXCR4 use, in the context of viruses from subtypes B and D (13). To determine whether CXCR4-using subtype C isolates also have distinctive V3 loop sequences, we isolated RNA from cultured isolates and sequenced their V3 regions, as described previously (6). Phylogenetic analysis of the nucleotide sequences of all 16 V3 loop sequences showed that all clustered with HIV-1 subtype C (data not shown), confirming the HMA data. The V3 loops of all the R5 viruses were 35 amino acids in length and contained the GPGQ motif at the crown that is characteristic of HIV-1 subtype C viruses (Fig. 1). A neutral serine residue was always present at position 11 and, in most cases, an amino acid with a negatively charged side chain (either D or E) was present at position 25. The net charge of the V3 loop was +3 to +5. In contrast, the V3 loops of the R5X4 and X4 viruses varied markedly from the consensus sequence, containing substitutions, deletions, and insertions. A common, and unusual, feature was a two-residue insertion between positions 13 and 14, most frequently isoleucine and glycine, which increased the length of the loop to 37 amino acids. A tetrapeptide motif containing positively charged arginine residues was also frequently present in the V3 loops of the R5X4 and X4 isolates, resulting in a marked increase in the overall positive charge of the loops to +6 to +9. Furthermore, all the R5X4 and X4 isolates had either a neutral or positively

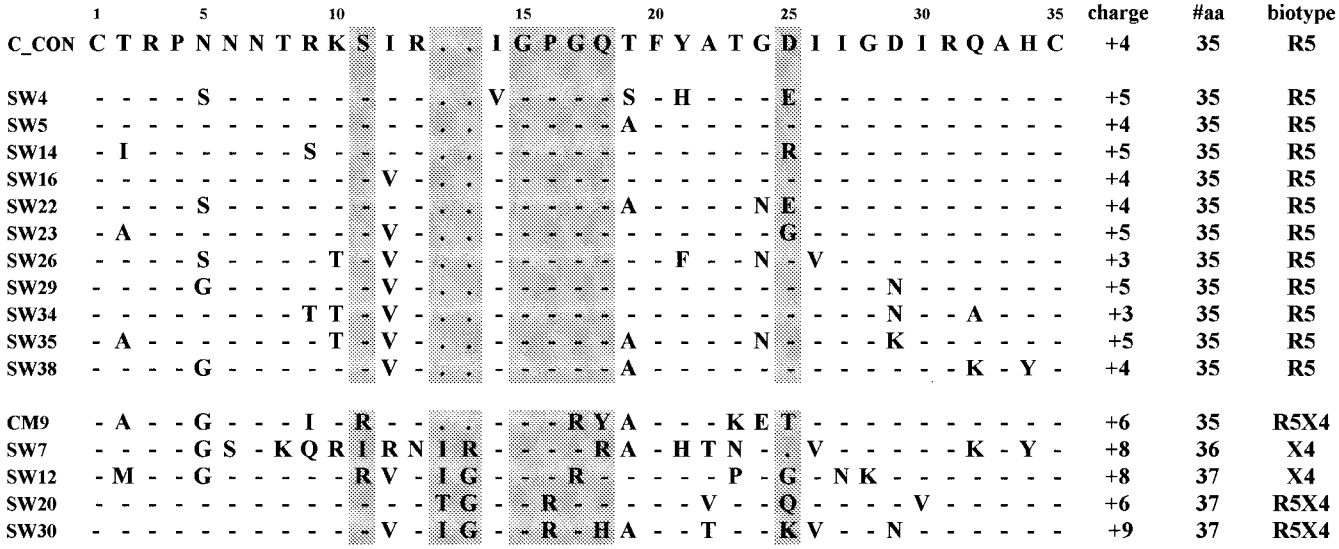

FIG. 1. Predicted V3 amino acid sequence of 16 South African HIV-1 subtype C isolates (12 R5 isolates and 5 R5X4 or X4 isolates) compared to an HIV-1 subtype C consensus sequence. The overall positive charge, number of amino acids, and biotype of each isolate are shown on the right. Dashes indicate concurrence, and a period indicates a deletion or lack of an insertion. Changed amino acids are indicated. Positions 11 and 25 (associated with changes in biological phenotype), the tip of the V3 loop, and the region with insertions are highlighted in grey.

charged amino acid substitution or a deletion at position 25. There were no sequence motifs that distinguished R5X4 from X4 viruses.

Discussion. Here we describe HIV-1 subtype C isolates from patients with late-stage AIDS that grow in MT-2 cells (SI viruses) and use CXCR4 for entry into cells. These isolates were fully sensitive to AMD3100, a CXCR4-specific entry inhibitor, and were able to grow in PBMC devoid of CCR5. Most of these SI viruses had significant genetic changes in the V3 loop compared to NSI viruses isolated from the same cohort. This observation is similar to those reported for viruses from subtypes B and D (13, 26). Hence, the HIV-1 subtype C envelope glycoproteins can accommodate the amino acid changes needed to use CXCR4 as a coreceptor. Probably, the gp120 proteins from all subtypes interact with CXCR4 in a broadly similar manner.

Our observations stand in contrast to those of previous reports that subtype C infections rarely involved viruses with the SI phenotype (1, 4, 7, 34, 38, 51). These earlier descriptions of subtype C isolates mostly involved cohorts of patients in the relatively early stages of HIV-1 infection, identified relatively soon after subtype C viruses started to spread rapidly in the geographic area under study. Now, we have deliberately selected for study patients with advanced AIDS, since they were more likely to harbor SI viruses. The isolation of CXCR4-using viruses from some of our late-stage AIDS patients suggests that subtype C viruses may undergo a phenotypic switch during disease progression, as occurs during subtype B infection (10, 41, 42). However, because our cohort was observational, we cannot know whether any of the patients with SI viruses had NSI viruses at an earlier stage. Prolonged, longitudinal followup of individuals with acute subtype C infection will be needed to address this issue. The viral phenotypes identified here are unlikely to be the result of in vitro selection pressure. Although viral stocks were grown in PBMC, the V3 sequences of cultured isolates were identical to those from the corresponding uncultured plasma samples (data not shown).

Although only 5 of our 29 (17%) AIDS patients were infected with SI viruses, this represents a higher frequency than was observed in previous studies on subtype C infection (1, 4, 7, 34, 38, 51). In subtype B cohorts, from 50 to 90% of patients with late-stage AIDS have been reported to harbor SI isolates (41, 42). Whether the frequency of subtype C X4 viruses will increase over time or will remain relatively low compared to that of subtype B infections remains to be determined. An analogy can be made to the HIV-1 subtype E epidemic in Thailand; there, early isolates were mostly of the NSI phenotype, but as the epidemic progressed, SI viruses started to appear with ever-increasing frequency (13, 30, 48, 49, 57). Further studies on subtype C infection may reveal the same trend. In any case, the assumptions that subtype C viruses use CCR5 almost exclusively are unlikely to be valid.

Most of the patients in our study had TB, which is the most common opportunistic infection in AIDS patients in South Africa. CCR5 expression levels are affected by environmental factors, such as TB, among African patients, which could favor a relatively high frequency of R5 viruses throughout the course of disease (9, 22). The transmission of R5 viruses may be particularly efficient in areas such as South Africa where there is a high incidence of early HIV-1 infection (24), which is associated with high viral loads and R5 viruses. Furthermore, any individuals who harbor SI viruses early in infection may not survive long enough in an African health care setting to become enrolled in a clinical cohort. Hence, this factor might lead to underrepresentation of SI isolates in a cohort such as ours. Patients with cryptococcal meningitis were particularly likely to have CXCR4-using viruses, which is probably related to the association between this opportunistic infection and advanced HIV-1 disease.

We previously showed that subtype C viruses were as sensitive as subtype B viruses to the CCR5 inhibitors, PRO 140, RANTES, and TAK-779 (52). Here, we have extended our observations to a more diverse group of viruses and we have also tested the CXCR4 inhibitor, AMD3100. Together, these studies support the clinical evaluation of coreceptor-specific entry inhibitors in South Africa; the antiviral responses are likely to be similar to those that have been observed in studies on subtype B infections in North America and Europe (Hendrix et al., 9th Conf. Retrovir. Opportun. Infect., abstr. 391-T, 2002; Reynes et al., 9th Conf. Retrovir. Opportun. Infect., abstr. 1). Subtype C accounts for the vast majority of global HIV-1 infections that are spread by heterosexual and perinatal transmission in developing countries. The ability of coreceptor inhibitors to prevent infection makes them particularly attractive as interventions in high-incidence settings. Novel approaches could include the use of entry inhibitors as microbicides to prevent sexual transmission or as supplements to be added to breast milk to block perinatal infection. Such interventions, however, would need to be effective, cheap, and simple to administer. Our data suggest that therapeutic or preventative approaches based on coreceptor entry inhibitors could be useful in developing countries where subtype C circulates and where such interventions are most desperately needed.

Nucleotide sequence accession numbers. The V3 sequences determined in this study were submitted to GenBank under the following accession numbers (isolate is identified in parenthesis): AY170657 (SW4), AY170658 (SW5), AY170659 (SW14), AY170660 (SW16), AY170661 (SW22), AY170662 (SW23), AY170663 (SW26), AY170664 (SW29), AY170665 (SW34), AY170666 (SW35), AY170667 (SW38), AF411967 (CM9), AF411966 (SW7), AY230878 (SW12), AY230879 (SW20), and AY230880 (SW30).

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