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Expanded Tropism of Primary Human Immunodeficiency Virus Type 1 R5 Strains to CD4^{+} T-Cell Lines Determined by the Capacity To Exploit Low Concentrations of CCR5

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Human immunodeficiency virus type 1 (HIV-1) non-syncytium-inducing (NSI) strains predominantly use the chemokine receptor CCR5, while syncytium-inducing (SI) strains use CXCR4. In vitro, SI isolates infect and replicate in a range of CD4^{+} CXCR4^{+} T-cell lines, whereas NSI isolates usually do not. Here we describe three NSI strains that are able to infect two CD4^{+} T-cell lines, Molt4 and SupT1. For one strain, a variant of JRCSF selected in vitro, replication on Molt4 was previously shown to be conferred by a single amino-acid change in the V1 loop (M.T. Boyd et al., J. Virol. 67:3649–3652, 1993). On CD4^{+} cell lines expressing different coreceptors, these strains use CCR5 predominantly and do not replicate in CCR5-negative peripheral blood mononuclear cells derived from individuals homozygous for Δ32 CCR5. Furthermore, infection of Molt4 and SupT1 by each of these three strains is potently inhibited by ligands for CCR5, including 2D7, a monoclonal antibody specific for CCR5. CCR5 mRNA was present in both Molt4 and SupT1 by reverse transcription-PCR, although CCR5 protein could not be detected either on the cell surface or in intracellular vesicles. The expanded tropism of the three strains shown here is therefore not due to adaptation to a new coreceptor but due to the capacity to exploit extremely low levels of CCR5 on Molt4 and SupT1 cells. This novel tropism observed for a subset of primary HIV-1 isolates may represent an extended tropism to new CD4^{+} cell types in vivo.

CD4^{+} T cells and macrophages are important cell targets of human immunodeficiency virus (HIV) infection. HIV strains have been classified into two main types: (i) syncytium-inducing (SI), T-cell line tropic (T-tropic), rapid/high strains and (ii) non-syncytium-inducing (NSI), macrophage-tropic (M-tropic), slow/low strains. In vitro, NSI viruses infect both macrophages and T-cell cultures but rarely T-cell lines. SI strains, however, replicate in a range of transformed CD4^{+} T-cell lines (31), while their capacity to infect macrophages is controversial (29, 38, 60, 66, 67, 81, 82). During primary acute infection, the majority of HIV type 1 (HIV-1) isolates are NSI (84), while SI strains emerge during disease progression in about 50% of AIDS patients (70). This emergence often precedes or coincides with a rapid decline in CD4^{+} cells in blood (41).

Two receptors are required on the surface of the target cell to trigger HIV entry: the CD4 receptor and a coreceptor (22, 28, 30). Coreceptors have seven transmembrane domains (7TM) and are either members of or related to the chemokine receptor family. More than 10 7TM receptors have been shown to act as coreceptors for entry of different HIV-1 strains in vitro (reviewed in references 4, 19, 26, and 47). All HIV-1 strains studied so far use either CCR5 or CXCR4 or both (67, 83). The discovery of HIV coreceptors has mainly explained the NSI/M-tropic versus SI/T-tropic phenotype, by showing that the former strains use CCR5 (1, 17, 22, 27, 28), a receptor for CC chemokines RANTES, MIP-1α, MIP-1β, and MCP-2 (21, 33, 54, 58), while the latter use CXCR4 (30), a receptor for the CXC chemokine stromal cell-derived factor 1 (10, 50). A new nomenclature for HIV strains has been adopted, so that isolates that use CCR5 are termed R5 viruses, those using CXCR4 are designated X4 viruses, and viruses able to use both coreceptors are called R5X4 viruses (5). CCR5 is predominantly expressed on macrophages (53, 73, 79, 82), dendritic cells (3, 9, 34, 82), brain microglial cells (32, 36, 62), and memory T cells (11) but absent on most T-cell lines, while CXCR4 is more widely expressed and present on both naive and memory T cells (11, 46). Thus, the cellular tropism of different strains of HIV-1 is largely determined by differential usage of chemokine receptors. However, this simple picture has several exceptions. Hence, some CCR5-dependent HIV-1 strains do not infect macrophages, although they express high levels of CCR5 (14, 23). Moreover, particular primary CXCR4-using strains do not replicate in several cell lines that express high levels of CXCR4 (43).

In this study, we show that while the majority of CCR5-using viruses do not infect T-cell lines, some strains (called Molt4/SupT1 strains) can infect the T-cell lines Molt4 and SupT1 (12). These strains include a molecularly cloned variant virus (C3) that was adapted in vitro for Molt4 replication and derived from JRCSF. A single amino acid change in the V1 loop accounts for C3's extended tropism for both Molt4 and SupT1 cells. The V3 loop on gp120 is a major determinant of both cell tropism (7, 15, 16, 37, 45, 61, 64, 72, 74) and more recently of coreceptor usage (8, 56, 68, 80). However, other envelope elements are also involved (39, 55, 56, 71), and several reports have implicated the V1 and V2 loops of gp120 (2, 12, 35, 40, 57, 69), which in addition to the required V3 domain influence the efficiency of replication of HIV-1 in primary macrophages (40, 63, 75) and in Jurkat T cells (13). Groenink et al. (35) described the configuration of a hypervariable locus in the V2 domain that appeared to be predictive for a switch from an NSI to an SI phenotype. V1 and V2 sequences act in conjunction with a CCR5-tropic V3 loop to confer CCR3 usage to some

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TABLE 1. Molt4 and SupT1 infection and coreceptor use by R5 strains[a]

HIV-1 strains	Infectivity								
	$TCID_{50}$/ml		FFU/ml						
			On U87/CD4 cells			On CCC/CD4 cells			
	On SupT1	On Molt4	CXCR4, CCR1, CCR2b	CCR3	CCR5	CCR8	GPR-15	STRL-33	GPR-1, CX3CR1, D6
C3	1.7	1	—[b]	—	3.5×10^4	—	—	—	—
C3/SupT1	1.7×10^1	1.7×10^1	—	—	7×10^5	—	—	20	—
JRCSF	—	—	—	—	4×10^3	—	—	—	—
E80	1	1	—	3×10^2	2.4×10^4	—	—	—	—
E80/SupT1	1.7×10^1	1.7×10^1	—	6×10^2	5×10^5	—	—	—	—
BR92	1×10^1	1.7	—	—	2.4×10^5	—	3.5×10^2	10	—
BR92/SupT1	1.7×10^3	1.7×10^2	—	—	2×10^6	—	5×10^2	10^2	—
BR53	—	—	—	—	5×10^3	—	—	—	—
BR90	—	—	—	—	1.2×10^5	—	—	—	—
SL2	—	—	—	4×10^2	3×10^5	—	3×10^2	10	—
SL3	—	—	—	—	2.2×10^4	—	—	—	—
SL4	—	—	—	—	2×10^3	—	—	—	—
JRFL	—	—	—	2×10^1	7×10^3	—	—	—	—
ADA	—	—	—	6×10^2	7×10^4	10^2	2×10^2	—	—

[a] U87 cells stably expressing human CD4 and either human CCR1, CCR2b, CCR3, CCR5, or CXCR4 as well as CCC/CD4 cells transfected with expression vectors encoding either human CCR8, GPR-15, STRL-33, GPR-1, CX3CR1, or D6 (49) were seeded into 48-well trays (Costar) at 6×10^4 cells per well. After 24 h, the cells were challenged with 100 μl of serial dilutions of NSI HIV-1 strains for 3 h at 37°C. After 4 days, the wells were fixed and immunostained as previously described (18). The number of positively stained foci was estimated by light microscopy, and the average number of FFU per milliliter was calculated from duplicate wells.

[b] —, no infection detected on SupT1 or Molt4 cells, and <10 FFU/ml on U87/CD4 or CCC/CD4 cells.

NSI strains (57). Kwong et al. (44) have recently reported the crystal structure of gp120 complexed with CD4 and a neutralizing antibody. This structure shows that the stems of the V1 and V2 loops and the V3 loops are located, respectively, on inner and outer domains of gp120 and on either side of a bridging sheet that spans these two domains. The coreceptor binding site is thought to contain amino acids in this bridging sheet and probably residues in the V3 loop. In some circumstances, the V1 and V2 loops are dispensible for high-affinity binding to coreceptors (77) and viral replication (13), yet when present on gp120 they can have a profound influence on tropism and coreceptor use.

In our study, we aimed to assess the coreceptor(s) used by the C3 variant of JRCSF that differs by a single amino acid in the V1 loop yet can infect the T-cell lines Molt4 and SupT1. We also assessed if the tropism of C3 for Molt4 and SupT1 cells reflected the phenotype of any unselected primary HIV-1 strains and may therefore represent tropism with in vivo relevance.

Replication of R5 viruses in Molt4 and SupT1 T-cell lines. We assessed if primary HIV-1 R5 strains passaged only in peripheral blood mononuclear cells could infect Molt4 or SupT1 cells. JRCSF and JRFL (42), ADA (74), and E80 (67) are previously described R5 strains. BR49, BR53, BR90, BR92, SL2, SL3, and SL4 are primary R5 isolates provided by St. Mary's Hospital, London, England. BR49, BR53, BR90, and BR92 were obtained from Brazilian patients (Infectious Disease Service, Porto Alegre, Brazil), while SL2, SL3, and SL4 were from asymptomatic patients from Thailand (Siriraj Hospital, Bangkok) (24, 67).

Of 10 NSI viruses tested, 2 strains (E80 and BR92) consistently replicated in Molt4 or SupT1; 8 other isolates failed to yield supernatant reverse transcriptase activity during 38 days culture. For one of these isolates, ADA, we prepared pseudotype virus that carried the vesicular stomatitis virus envelope glycoprotein G. This pseudotype efficiently infected both Molt4 and SupT1, thus confirming that the block to infection occurred early in the replication cycle and could be bypassed by virions carrying a foreign envelope glycoprotein.

The efficiency of E80 and BR92 as well as the C3 variant of JRCSF to infect SupT1 or Molt4 cells was assessed by estimating endpoint infectivity titers (expressed as 50% tissue culture infective dose [$TCID_{50}$] per milliliter) (Table 1). These were lower than titers for U87/CD4/CCR5 cells, which express high cell surface concentrations of CCR5. When stocks of E80, BR92, or the C3 variant were prepared from and retitrated back on SupT1 or Molt4 cells, slightly higher titers were noted. For instance, over an endpoint titer of 10^3 $TCID_{50}$/ml was observed for BR92 passaged through SupT1 cells. However, these viruses also had higher infectivity titers for U87/CD4/CCR5 cells; therefore, there was no convincing evidence of further adaptation for SupT1 and Molt4 replication (Table 1).

Coreceptor use of Molt4/SupT1 R5 strains. It was possible that Molt4 and SupT1 infection was due to the capacity of these strains to use a novel Molt4/SupT1 coreceptor. We therefore tested the coreceptors that were used by each strain. To determine the coreceptor usage of the isolates studied, we challenged U87 cells stably expressing human CD4 and either human CCR1, CCR2b, CCR3, CCR5, or CXCR4 with HIV-1 strains and monitored infection after 4 to 5 days by immunostaining with an anti-p24 antibody as the primary antibody, followed by incubation with a secondary antibody conjugated to β-galactosidase as described previously (18). For other coreceptors, CCC/CD4 cells were transiently transfected with either CCR8, GPR-15, STRL-33, GPR-1, CX3CR1, or D6 as previously described (67). Table 1 shows infectivity titers in focus-forming units (FFU) per milliliter. JRCSF and C3 infected only cell lines expressing CCR5. All the other M-tropic viruses efficiently infected $CCR5^+$ cells but additionally utilized one or more of the following coreceptors, albeit less efficiently: CCR3 (E80, SL2, ADA, and JRFL), GPR-15 (BR92, SL2, and ADA), STRL-33 (BR92 and SL2), and CCR8 (ADA). None of the viruses used CXCR4 on either U87/CD4 or GHOST/CD4 cells (data not shown). Thus, there was no correlation between Molt4 or SupT1 infection and the use of a particular coreceptor, except for CCR5. Moreover, none of these strains were able to replicate in peripheral blood mono-

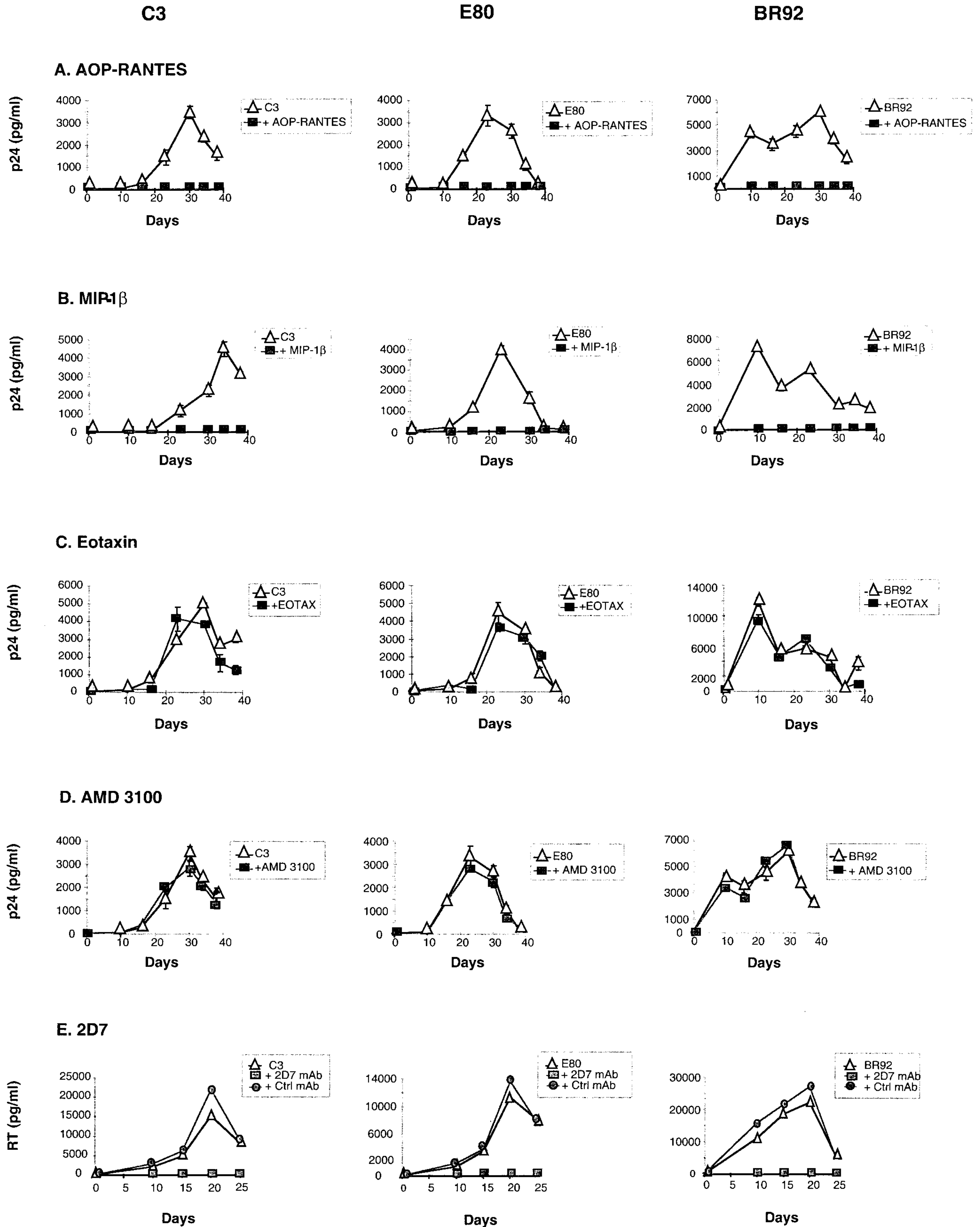

FIG. 1. Inhibition of C3, E80, and BR92 infection by chemokine receptor ligands. SupT1 and Molt4 cells were treated with virus alone (triangles) or virus plus chemokine receptor ligand (AOP-RANTES, MIP-1β, eotaxin, AMD3100, or the CCR5-specific MAb 2D7, as indicated) (squares). p24 antigen or RT activity (E) in supernatants was measured every 3 to 4 days as previously described (65, 67). For 2D7 inhibition, a control anti-CXCR4 antibody was also tested (circles).

nuclear cells from patients homozygous for the CCR5 Δ32 deletion (data not shown).

Inhibition of Molt4 and SupT1 infection by using coreceptor ligands. To assess the coreceptors used by C3, E80, and BR92 for SupT1 or Molt4 infection, we tried to inhibit infection by using various coreceptor ligands, including chemokines, modified chemokines, monoclonal antibodies (MAbs), and small organic molecules (reviewed in reference 52). AOP-RANTES is a chemically modified form of RANTES and a potent inhibitor of infection via CCR5 (65). We also tested RANTES itself as well as MIP-1β (20) and eotaxin, a chemokine specific for CCR3 (76). To assess if CXCR4 was involved, we tested inhibition by AMD3100, a bicyclam that is a potent and specific inhibitor of CXCR4 infection (25, 59). SupT1 and Molt4 cells were seeded at 10^5 cells per well in 96-well trays; 50 μl of medium containing an appropriate chemokine receptor ligand was added at twice the final concentration and incubated at 37°C for 30 min; 50 μl of virus was then added, and the medium was incubated for 3 h at 37°C. The cells were washed four times, and fresh medium containing the relevant chemokine at the required concentration (500, 1,500, or 3,000 ng/ml) was added. For the time course experiment, supernatants were harvested every 3 to 4 days from days 0 to 40, and fresh medium containing appropriate ligands was added.

The results for chemokine receptor ligands used on SupT1 are shown in Fig. 1A to D. Similar results were obtained on Molt4 (data not shown). AOP-RANTES (Fig. 1A) and MIP-1β (Fig. 1B) completely inhibited the replication of C3, E80, and BR92, as did RANTES (data not shown), while eotaxin (Fig. 1C) and AMD3100 (Fig. 1D) had no effect. Although RANTES and MIP-1β bind CCR5, they also bind other 7TM receptors that are potential coreceptors. For instance, MIP-1β binds CCR8 (6) and D6 (49) as well as CCR5. However, C3, E80, and BR92 do not use either as coreceptor (Table 1).

To confirm that C3, E80, and BR92 used CCR5 to infect Molt4 and SupT1 cells, we tested inhibition by using 2D7, a MAb specific for CCR5 that was previously used to block R5 entry or infection in other studies (73, 78). Complete inhibition of replication was observed by 2D7 used at 20 μg/ml but not by a control MAb, used at the same concentration, that recognized CXCR4 (Fig. 1E). Thus, data shown in Fig. 1A, B, and D indicate that CCR5 is the coreceptor used for infectivity of Molt4 and SupT1 cells, even though it could not be detected on the cell surface or internally by immunofluorescence (data not shown).

A single amino acid change in the V1 loop of JRCSF allows the C3 variant to enter and replicate in Molt4 and SupT1 but not several other T-cell lines (12). Two primary R5 isolates out of ten also infected Molt4 and SupT1, providing evidence that viruses like the C3 variant do exist in vivo. In this study, we aimed to investigate whether Molt4/SupT1 tropism was conferred by the use of a specific coreceptor. Our results show that several CCR5 ligands including 2D7 (a MAb specific for CCR5) blocked infection of Molt4 and SupT1 cells, indicating that these strains were able to exploit undetectable levels of CCR5 on these cell lines. Most of the M-tropic CCR5-using strains tested could not infect Molt4 or SupT1 cells, indicating that the use of CCR5 as a coreceptor does not accurately predict the cell tropism of any particular HIV-1 strain. In other systems, receptor expression level has been shown to influence virus entry (48, 79). For instance, the concentrations of CD4 and CCR5 required for efficient infection by R5 viruses are interdependent and the requirement for either is increased when the other is limiting (51). CCR5 expression is variable in vivo (46). Moreover, JRCSF was unable to produce infection in culture when less than 2% of the cells expressed CCR5 (79).

A striking point is that although CCR5 mRNA was present in both Molt4 and SupT1 cells, the protein was not clearly identified, either on the cell surface or internally in permeabilized cells (data not shown). This presumably reflects a very low level of expression, although we cannot rule out that a different conformation of CCR5 or yet unidentified factors impair detection by interfering with the binding of the CCR5-specific MAbs. The CCR5 cDNAs obtained from mRNA extracted from Molt4 and SupT1 were sequenced and found to be 100% homologous to the GenBank sequence. Furthermore, neither of these cell lines produced significant amounts of β-chemokines in the cell supernatant (data not shown). Thus, C3, E80, and BR92 are able to exploit apparently undetectable levels of CCR5 on Molt4 and SupT1 to trigger entry into cells whereas other strains cannot.

To conclude, our results show that a small subset of primary HIV-1 R5 strains are able to infect $CD4^+$ T-cell lines, Molt4 and SupT1. These strains do not use an alternative coreceptor but are able to exploit low concentrations of CCR5 for infection. Molt4/SupT1 tropism therefore identifies primary HIV R5 strains that are likely to have an expanded or altered tropism for $CD4^+$ cells in vivo.

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