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Use of alternate coreceptors on primary cells by two HIV-1 isolates

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Abstract

Two HIV-1 isolates (CM4 and CM9) able to use alternate HIV-1 coreceptors on transfected cell lines were tested for their sensitivity to inhibitors of HIV-1 entry on primary cells. CM4 was able to use CCR5 and Bob/GPR15 efficiently in transfected cells. The R5 isolate grew in Δ32/Δ32 CCR5 PBMC in the absence or presence of AMD3100, a CXCR4-specific inhibitor, indicating that it uses a receptor other than CCR5 or CXCR4 on primary cells. It was insensitive to the CCR5 entry inhibitors RANTES and PRO140, but was partially inhibited by vMIP-1, a chemokine that binds CCR3, CCR8, GPR15 and CXCR6. The coreceptor used by this isolate on primary cells is currently unknown. CM9 used CCR5, CXCR4, Bob/GPR15, CXCR6, CCR3, and CCR8 on transfected cells and was able to replicate in the absence or presence of AMD3100 in Δ32/Δ32 CCR5 PBMC. It was insensitive to eotaxin, vMIP-1 and I309 when tested individually, but was inhibited completely when vMIP-1 or I309 was combined with AMD3100. Both I309 and vMIP-1 bind CCR8, strongly suggesting that this isolate can use CCR8 on primary cells. Collectively, these data suggest that some HIV-1 isolates can use alternate coreceptors on primary cells, which may have implications for strategies that aim to block viral entry.

Keywords: HIV-1; Alternate coreceptors; CCR8; Bob/GPR15

Introduction

The human immunodeficiency virus type 1 (HIV-1) gains entry into a cell by binding to host cell surface receptors. This interaction is a sequential process and can be defined in three stages: namely, CD4 binding, coreceptor binding and the fusion process. The two most important coreceptors are CCR5 and CXCR4, and all HIV-1 isolates tested to date use one or both (Doms and Moore, 2000). A few rare isolates can also use other coreceptors present on coreceptor-transfected cell-lines, including CCR1, CCR2b, CCR3, Bob/GPR15, CXCR6 (STRL33) and CCR8 (Cilliers et al., 2003; Deng et al., 1997; Edinger et al., 1998; Pohlmann et al., 1999; Xiao et al., 1998). Some of these receptors such as CXCR6 and CCR8 have been shown to function as HIV entry coreceptors on primary cells in vitro (Lee et al., 2000; Sharron et al., 2000; Zhang et al., 2000, 2001). Further evidence that some isolates can use alternate coreceptors comes from studies where both major coreceptors are either absent or blocked. In this regard, the use of PBMC with a 32-base pair deletion in CCR5 (Δ32/Δ32 CCR5) in the presence of a CXCR4 inhibitor has proven particularly useful (Aze-

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vedo-Pereira et al., 2003; Willey et al., 2003; Zhang et al., 2000). Identification of alternative HIV-1 coreceptors is hampered by the lack of suitable ligands although in some instances chemokines have been used to provide suggestive evidence (Willey et al., 2003). Collectively, these data demonstrate that some rare isolates can exploit alternate coreceptors on primary cells in vitro, although whether such receptors are used by HIV-1 in vivo remains unclear (Sharron et al., 2000; Willey et al., 2003; Zhang and Moore, 1999; Zhang et al., 2001).

The ability of isolates to use particular coreceptors for viral entry can be determined by the use of inhibitors that block specific receptors (Moore et al., 2004). Inhibitors of CCR5 and CXCR4 have been shown to be highly effective in vitro and are being developed for clinical use (Moore and Doms, 2003; Seibert and Sakmar, 2004). Inhibitors binding to CCR5 include PRO140, a monoclonal antibody (Mab) directed at CCR5, and RANTES, a natural ligand for CCR5 (Olson et al., 1999; Wu et al., 1997). AMD3100 is a small molecule inhibitor able to bind to CXCR4 and prevent CXCR4-using HIV-1 isolates from infecting cells (Donzella et al., 1998; Schols et al., 1997). Blocking CCR5 is of particular interest as this is the coreceptor used during the initial phases of HIV-1 infection and R5 viruses can persist until the end stage (Douek et al., 2003; Moore et al., 2004). The ability of some viral isolates to use alternate coreceptors could impact on the efficacy of entry inhibitors and possibly also HIV pathogenesis. Hence, it is relevant to identify and characterize HIV isolates able to use alternate coreceptors on primary cells.

A previous study reported that viral macrophage inflammatory protein-1 (vMIP-1), a promiscuous chemokine expressed by Human Herpes Virus 8, could bind to a range of coreceptors, including CCR8, GPR1, CXCR6 and to a lesser extent Bob/GPR15 (Dairaghi et al., 1999; Willey et al., 2003). This chemokine has been shown to prevent HIV-1 infection of some isolates on primary cells when the major coreceptors are absent or blocked. This suggests that vMIP-1 might be useful in identifying alternate coreceptors that could be used by HIV-1 isolates (Willey et al., 2003). The aim of this study was to further investigate how two unusual HIV-1 isolates can use alternate coreceptors on primary cells, by using chemokines with defined receptor binding profiles.

Results

Coreceptor usage of CM4 and CM9

Two HIV-1 isolates from patients with cryptococcal meningitis, a severe AIDS defining condition, were selected for this study. CM9 is an HIV-1 subtype C R5X4 isolate able to use CCR5, CXCR4, CCR3, CXCR6 and Bob/GPR15 to replicate in transfected cell lines (Cilliers et al., 2003) (Table 1). While the efficiency with which CM9 used the three minor coreceptors was considerably lower than for the two major coreceptors (i.e. CCR5 and CXCR4), their usage was highly reproducible on GHOST.3 cell lines. CM4, a complex recombinant R5 virus with subtype C-like envelope was able to use both CCR5 and Bob/GPR15 with equal efficiency on transfected cell lines (Table 1). This isolate was unable to use CXCR4, as judged by its inability to replicate in U87.CD4.CXCR4 or MT-2 cells. It was also unable to use CCR3, CXCR6 or CCR1. CCR1 was included as a negative control for both CM4 and CM9 (Table 1).

Table 1
Growth of CM4 and CM9 on coreceptor transfected cell lines

Isolate	Biotype	Coreceptor usage (day 12 p24 ng/ml)				
		U87.CD4		GHOST.3		
		CCR5	CXCR4	Bob/GPR15	CCR3	CXCR6
CM4	R5	70	<1	65	<1	<1
CM9	R5X4	69	48	3	3	8.5

Note. Replication levels between U87.CD4 cells and GHOST.3 cells may not be directly comparable.

Both CM4 and CM9 were able to replicate in Δ32/Δ32 CCR5 PBMC efficiently although CM4 replicated to higher levels (84 ng/ml versus 10 ng/ml p24 in one representative experiment) (see also Figs. 2 and 3). The growth seen for CM9 in Δ32/Δ32 CCR5 PBMC is most likely related to the ability of this isolate to use CXCR4 (Cilliers et al., 2003). Since CM4 is unable to use CXCR4, growth in Δ32/Δ32 CCR5 PBMC must be due to an alternate receptor. Whether CM4 is using Bob/GPR15 to enter PBMC cannot be assessed at present as no inhibitor, including polyclonal antibodies, or natural ligands to block this coreceptor is available.

Use of CCR5 and CXCR4 inhibitors to determine coreceptor use

To further define coreceptor specificity of CM9 and CM4, specific inhibitors were used. The CCR5 inhibitors included the anti-CCR5 Mab PRO140 and the CC-chemokine RANTES. AMD3100 was also used to block CXCR4. The assays were performed on wt/wt CCR5 PHA-stimulated PBMC with a p24 antigen read-out on day 6. CM4 was not inhibited by either PRO140 or RANTES at the same concentrations that were active against other HIV-1 isolates (Table 2) (Cilliers et al., 2003; Trkola et al., 2001). This isolate was also insensitive to AMD3100, consistent with its inability to use CXCR4. Hence, neither CCR5 nor CXCR4 inhibitors were effective against CM4. In contrast, CM9 was sensitive to AMD3100 (97% inhibition on day 6, Table 2). However, like CM4, CM9 was insensitive to the CCR5 inhibitors. Thus, the major coreceptor of preference for this pleiotropic isolate is CXCR4 (Cilliers et al., 2003).

We next tested the ability of CM4 and CM9 to be inhibited by the fusion inhibitor Enfuvirtide, which is not

Table 2
Effect of CCR5, CXCR4 and fusion inhibitors on CM4 and CM9 replication in (wt/wt) CCR5 PBMC

Isolate	Biotype	% inhibition			
		PRO140 (167 nM)	RANTES (19 nM)	AMD3100 (500 nM)	Enfuvirtide (1 μg/ml)
CM4	R5	33	24	4	**99**
CM9	R5X4	36	34	**97**	**100**

coreceptor-specific. Both isolates were fully sensitive to Enfuvirtide and were inhibited by >99% at 1 μg/ml (Cilliers et al., 2004). This demonstrates that use of an alternate coreceptor does not radically alter the envelope-mediated fusion process.

Env sequence analysis of CM4 and CM9 grown in different cell lines

To determine if minor quasispecies were accounting for multiple coreceptor use, CM4 and CM9 were grown in different cell lines. The *env* genes were then sequenced and compared to those of the original infecting strain. CM4-P2 was grown in normal donor PBMC, Δ32/Δ32 CCR5 PBMC, and GHOST.3 cells expressing Bob/GPR15. The amino acid sequences of the expanded viruses were then compared to the original infecting stock (CM4-P1). All gp160 sequences from CM4-P2 grown in different cells were identical (Fig. 1A). When these sequences were compared to the earlier stock (CM4-P1), only a single amino acid change in the V3 region (T300N) was noted (Fig. 1A). Hence, the major viral population of CM4 uses both CCR5 and Bob/GPR15 as coreceptors on transfected cell lines as well as on Δ32/Δ32 CCR5 PBMC, suggesting that the same envelope glycoprotein configuration can use both CCR5 and Bob/GPR15. CM4 had a low overall charge of +3 in the V3 loop, which is more consistent with usage of CCR5 than of CXCR4 (Hoffman et al., 2002).

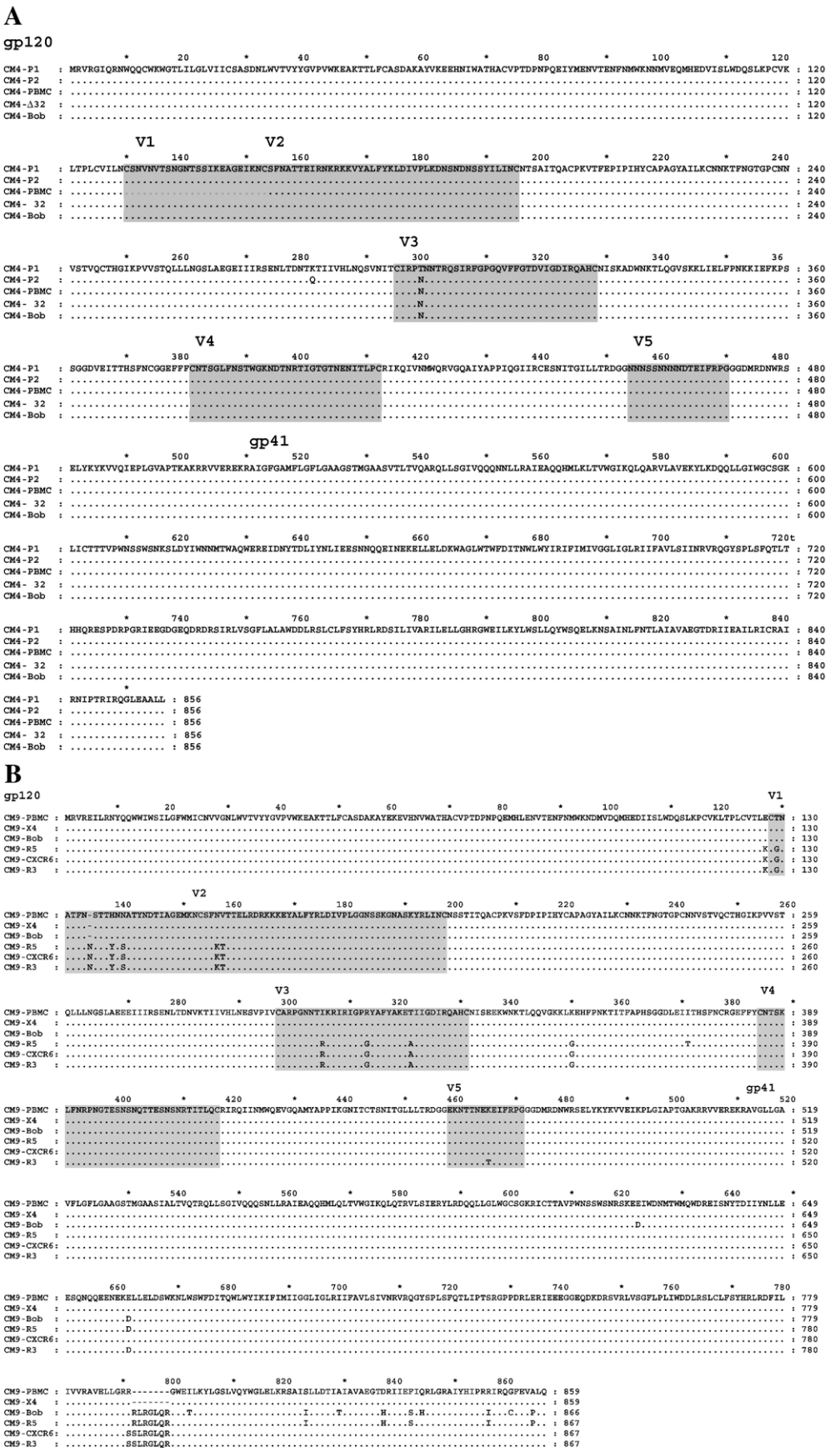
A similar analysis was done using CM9 grown in cell lines expressing CCR5, CXCR4, CCR3, CXCR6 and Bob/GPR15, with the resulting sequences then compared to the original isolate grown in wt/wt PBMC. Distinct amino acid sequence changes in gp160 were noted between the selected populations, particularly in the V1/V2 and V3 regions of gp120, and in the cytoplasmic tail of gp41 (Fig. 1B). Viruses that grew in the CXCR6/Bonzo and CCR3 cell lines were virtually identical in sequence, and most similar to the viruses that grew in the CCR5 transfected cells. The sequence of the virus grown in CXCR4-expressing cells was identical to the original PBMC sequence and most similar to the virus that grew out of the Bob/GPR15 cells. Three changes were noted in the V3 crown region between these two populations. The viruses expanded on the CXCR4 or Bob/GPR15 cells had a GPRY motif while those grown in CCR5, CXCR6/Bonzo or CCR3 expressing cells had a GPGY sequence. There was no difference in the overall charge (+6) or number of amino acids in the V3 region of the different selected populations, so no prediction about coreceptor usage could be made from the genotype. An insertion in the cytoplasmic tail of the gp41 region was noted for almost all viruses grown in coreceptor-transfected cells except for those grown in CXCR4 transfected cells (Fig. 1B). This suggests that the original CM9 isolate comprised at least 2 distinct populations, one that uses CCR5, CXCR6 and CCR3 and a second that uses CXCR4 and Bob/GPR15. This mixed population may account for the usage of at least 2 different coreceptors by this isolate.

Inhibition assays on Δ32/Δ32 CCR5 PBMC

We next explored the ability of chemokines that act as natural ligands for alternate coreceptors to inhibit CM4 and CM9 replication in Δ32/Δ32 CCR5 PBMC. The chemokine vMIP-1 is a promiscuous chemokine secreted by Human Herpes Virus 8 that binds to CCR8, GPR1, Bob/GPR15 and CXCR6 (Dairaghi et al., 1999; Simmons et al., 2000; Willey et al., 2003). Eotaxin is the ligand for CCR3, and I309 is specific for CCR8. CM9 was grown in Δ32/Δ32 CCR5 PBMC in the presence of vMIP-1, I309 and eotaxin either singly or in combination with AMD3100. It was previously shown that CM9 could be inhibited by AMD3100 for up to 6 days on normal donor PBMC, consistent with its efficient use of CXCR4 for entry (Cilliers et al., 2003). However, in this study, we found that CM9 was eventually able to replicate in the presence of AMD3100 on Δ32/Δ32 CCR5 PBMC when the assay was extended beyond 6 days (Fig. 2A). When AMD3100 was combined with eotaxin, the natural ligand for CCR3, there was enhanced viral replication. vMIP-1 and I309 had no effect on CM9 replication when tested individually (Fig. 2A) but when they were combined with AMD3100, inhibition was complete (Fig. 2B). Since both I309 and vMIP-1 can bind to CCR8, CM9 may be using CCR8 to infect Δ32/Δ32 CCR5 PBMC in the presence of AMD3100. This was confirmed when CM9 was grown on NP2/CD4 cell lines expressing CCR8 (Fig. 4). Replication in these cells was as efficient as growth in NP2/CD4 cells expressing either CCR5 or CXCR4.

CM4 was tested in Δ32/Δ32 CCR5 PBMC in the absence and presence of AMD3100, vMIP-1, I309 and CXCL16

Fig. 1. (A) Full-length envelope amino acid sequence alignment of CM4 grown in different cell lines and PBMC. The original culture supernatant is labeled CM4-P1. The regrown strain is labeled CM4-P2. CM4-P2 grown in PBMC (CM4-PBMC), Δ32/Δ32 CCR5 PBMC (CM4-Δ32) and GHOST.3.Bob cell line (CM4-Bob) is shown. The variable loops are highlighted in grey and amino acid changes are shown. Dots indicate identical amino acids. (B) Full-length envelope amino acid sequence of CM9 grown in different cell lines and PBMC (CM9-PBMC). CM9 was grown in U87.CD4.CXCR4 (CM9-X4), GHOST.3 transfected with Bob/GPR15 (CM9-Bob), U87.CD4.CCR5 (CM9-R5) and GHOST.3.CXCR6 (CM9-CXCR6) and CCR3 (CM9-R3), respectively. The variable loops are highlighted in grey and amino acid changes are shown. Dots indicate identical amino acids and dashes indicate amino acid insertions.


A
gp120
V1
V2
V3
V4
V5
gp41
CM4-P1
CM4-P2
CM4-PBMC
CM4-Δ32
CM4-Bob
B
gp120
V1
V2
V3
V4
V5
gp41
CM9-PBMC
CM9-X4
CM9-Bob
CM9-R5
CM9-CXCR6
CM9-R3

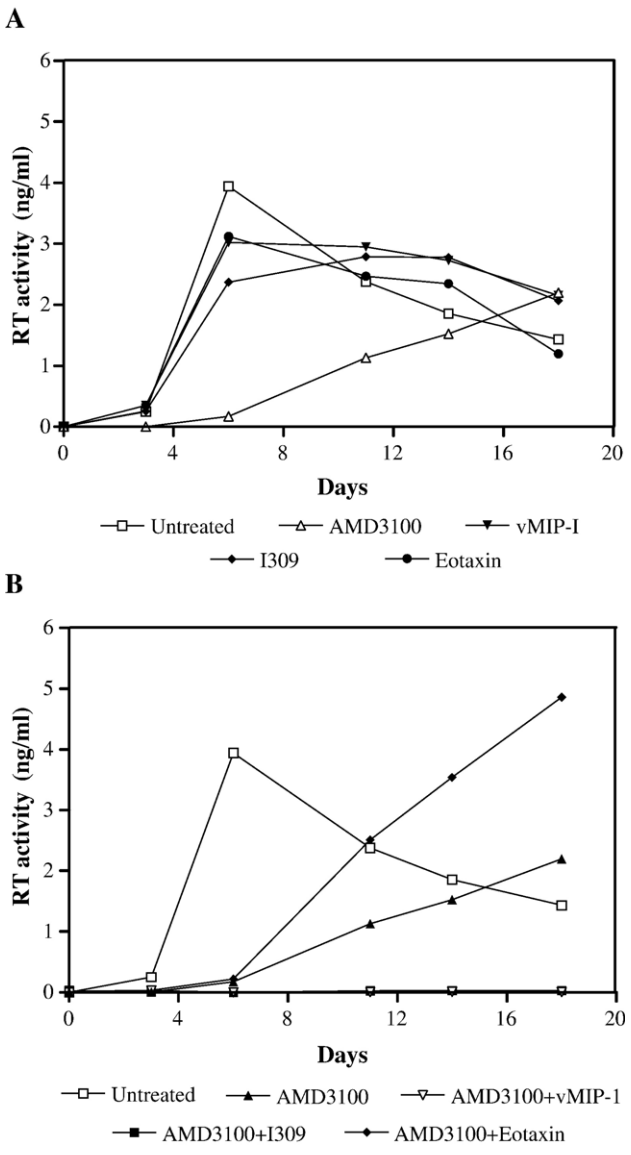


Fig. 2. Inhibition of CM9 in Δ32/Δ32 CCR5 PBMC. (A) Growth curve of CM9 in the presence of AMD3100, vMIP-1, I309, eotaxin or without inhibitors. (B) CM9 in the presence of AMD3100 alone or in combination with vMIP-1, I309 or eotaxin or no inhibitors. RT activity was used to measure viral replication levels.

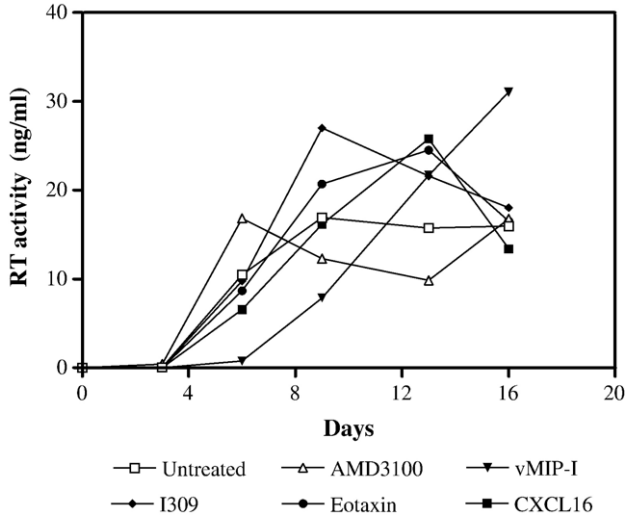


Fig. 3. Replication of CM4 in Δ32/Δ32 CCR5 PBMC in the presence of AMD3100, vMIP1, I309, eotaxin, CXCL16 or without inhibitors. RT activity was used to measure viral replication levels.

(Fig. 3). CXCL16 is the natural ligand for CXCR6. None of these inhibitors had any effect on CM4 replication except vMIP-1, which caused a delay. Hence, CM4 is using a coreceptor other than CCR5, CXCR4, CCR8 or CXCR6 to productively infect PBMC. Its identity is still unclear, but it may be a vMIP-1 receptor.

Discussion

The identification of HIV-1 isolates able to use coreceptors other than CCR5 and CXCR4 on primary cells is rare (Lee et al., 2000; Willey et al., 2003; Zhang et al., 2000). While the significance of alternate coreceptor usage is uncertain, the possibility that HIV-1 can expand its cellular host range through the use of alternate coreceptors remains a theoretical possibility especially for HIV-1 subtype C where CXCR4 is rare. Here, we report on how two isolates, CM4 and CM9, can use alternate coreceptors to infect primary cells. Inhibition data suggest that CCR8 and an as yet unknown coreceptor can mediate HIV-1 entry for these two isolates, respectively. Such isolates would be expected to be refractory to inhibition by agents directed at CCR5 and CXCR4.

CM4, an R5 isolate able to utilize Bob/GPR15, on transfected cell lines was isolated from a patient with advanced HIV disease. Its insensitivity to both CCR5 and CXCR4 inhibitors suggested that it could use an alternate coreceptor to infect PBMC. This receptor is apparently also present on Δ32/Δ32 CCR5 PBMC and can support high levels of replication. Whether CM4 is able to use Bob/GPR15 on primary cells remains to be determined. Bob/GPR15 is an orphan G-protein coupled receptor whose mRNA is widely expressed in the colon, spleen, PHA stimulated PBMC, purified T-cells and to a lesser extent in unstimulated PBMC (Deng et al., 1997). Until a natural ligand to Bob/GPR15 is identified, it will remain difficult to prove that CM4 is using this receptor to enter primary cells. In a previous study, a different batch of CM4 (CM4-P1) was inhibited by RANTES, PRO140 and TAK779 suggesting that entry was CCR5-dependent (Trkola et al., 2001). For the experiments reported here, CM4 had been expanded in PHA-stimulated PBMC to generate a high titer stock (CM4-P2). The entry of this virus preparation was found to be independent of CCR5, as reflected by its ability to grow in Δ32/Δ32 CCR5 PBMC. Experiments directly comparing the properties of the original (CM4-P1) and expanded viral stock (CM4-P2) confirmed these findings (data not shown). The envelope sequences of CM4-P1 and CM4-P2 showed

that they were more than 99% identical (Fig. 1A). It is unlikely that the single amino acid change T300N between CM4-P1 and CM4-P2 is responsible for the altered coreceptor phenotype. It is more likely that the ability of CM4-P2 to grow in Δ32/Δ32 CCR5 PBMC is related to the viral titer, the ability to use alternate coreceptors being only revealed when the viral titer is high. Nevertheless, we cannot formally rule out that the ability of CM4 to replicate in Δ32/Δ32 CCR5 PBMC, presumably by alternate coreceptor-use, was acquired or selected for during in vitro passage.

CM9, an R5X4 virus, was also isolated from a patient with advanced HIV infection and it too is a pleiotropic isolate that has a preference for CXCR4-usage (Cilliers et al., 2003). Other studies have shown that CXCR4 is the coreceptor of preference for most R5X4 isolates, which explains why many are completely inhibited by AMD3100 (Yi et al., 1999). CM9 was previously shown to use alternate coreceptors, CCR3, CXCR6 and Bob/GPR15 albeit less efficiently than CXCR4 (Cilliers et al., 2003). In this study, we show that in addition to using these five coreceptors, CM9 can also use CCR8. This was confirmed using NP2/CD4/CCR8 transfected cells, which supported CM9 replication to high levels (Fig. 4). However, the use of CCR8 appeared to be less efficient on PBMC compared to transfected cell lines, similar to CCR5 usage by this isolate. Thus, during the first 6 days, replication of CM9 in PBMC was fully inhibited by AMD3100, suggesting a dominant role of CXCR4. However, replication at later time points was inhibited by both vMIP-1 and I309, a CCR8-specific inhibitor, in the presence of AMD3100. Thus, it appears that growth in the presence of AMD3100 after 6 days is due to the ability of this isolate to use CCR8. This later use of CCR8 may be related to expression levels of this receptor on PBMC, which may vary in culture, or the utilization of CCR8 by different populations in the CM9 stock, or less efficient usage of CCR8 compared to CXCR4. CCR8 is expressed on primary lymphocytes and at high level in the thymus and has the ability to facilitate HIV-1 entry into thymocytes (Baecher-Allan et al., 2001; Dieckmann et al., 2001; Iellem et al., 2001; Lee et al., 2000). This receptor has been reported to be used by a handful of simian immunodeficiency virus (SIV), HIV-1 and HIV-2 isolates and is one of the more commonly used alternate coreceptors (Horuk et al., 1998; Rucker et al., 1997). However, most of the above studies were performed only on cell lines, raising questions as to the significance of the findings for understanding replication in primary cells. Our finding that CM9 uses CCR8 on primary cells suggests that CCR8 may indeed prove to be a significant HIV-1 coreceptor for some isolates, a factor that may need to be taken into consideration in clinical studies of CCR5 and CXCR4 inhibitors. Chemokines that target CCR8, such as I309 may need to be considered for drug development.

Analysis of envelope sequences from viruses grown in PBMC and cell lines revealed that the same viral population of CM4 was able to use CCR5, Bob/GPR15 and a coreceptor on CCR5 deficient PBMC. In contrast, distinct viral populations may account for some of the promiscuous coreceptor activity of CM9. Two distinct viral populations could be discerned in gp160 from CM9. The population able to use CCR5, CXCR6 or CCR3 had three amino acid changes in the V3 region and 4 amino acid changes in V2 compared to the population able to utilize CXCR4 or Bob/GPR15. However, the population able to utilize Bob/GPR15 also had an insertion in the gp41 similar to that found in the CCR5, CXCR6 and CCR3-using virus. This suggests that the Bob/GPR15 utilizing envelope is distinct from all the other populations; it even may be a recombinant between different populations. Further studies on this virus may provide clues as to the genetic determinants of both major and alternate coreceptor usage. The CCR8-using virus is likely to be a subpopulation different than the one that uses CXCR4, as the usage of the latter coreceptor was effectively inhibited up to day 6 in a PBMC culture. Hence, the sequence analysis performed on CM9 grown in the different cell lines may not be used to conclusively indicate which viral population accounts for use of the CCR8 receptor on Δ32/Δ32 CCR5 PBMC.

Whether it is significant that both the CM4 and the CM9 isolates were derived from patients with cryptococcal

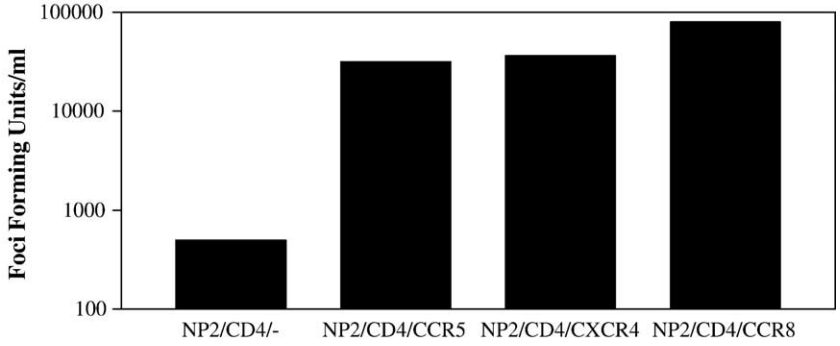


Fig. 4. Replication of CM9 in human glioma NP-2 cell line expressing CD4 with either CCR5, CXCR4 or CCR8. Cells were infected and stained with anti-p24 monoclonal antibody and a B-galactosidase conjugated secondary antibody. Infected cells were stained with X-gal and the number of infectious foci counted. Mean of 2 independent experiments.

meningitis still remains unclear. Cryptococcal meningitis is a severe AIDS condition that arises after the immune system has deteriorated and it is associated with high levels of viral replication in blood and cerebrospinal fluid (Morris et al., 1998). It is possible that when CD4 target cells are limited, HIV-1 isolates exploit other receptors to maintain high levels of replication. Alternatively, cryptococcal meningitis could up-regulate other chemokine receptors or allow access of HIV-1 to other cell populations, such as in the central nervous system that may promote use of alternate coreceptors. These data may also point to the need for the use of a combination of entry inhibitors that target different stages in the entry cycle, such as the CD4-binding site or the fusion process. Enfuvirtide was able to efficiently inhibit both CM4 and CM9 strains suggesting that it is not influenced by alternate coreceptor usage. Thus, ENF may be a useful adjunct to coreceptor inhibitors (Tremblay et al., 2000, 2002). Collectively, these data suggest that alternate coreceptors need to be considered when developing entry inhibitors and that such isolates pose a threat to the clinical use of inhibitors targeting only CCR5 and CXCR4.

Materials and methods

HIV-1 viral isolates

Two previously described isolates, CM4 (99ZACM4) and CM9 (99ZACM9) from a cohort of AIDS patients, were selected for further analysis (Bredell et al., 2002; Cilliers et al., 2003; Papathanasopoulos et al., 2002; Trkola et al., 2001). CM4 was isolated in 1999 from a 34-year old male in Johannesburg, South Africa who had advanced HIV infection and cryptococcal meningitis. This patient had a viral load of 163,755 HIV-1 RNA copies/ml (by bDNA assay) and CD4 count of 47 cells/μl. CM9 was isolated in 1999 from a 30-year old female also with cryptococcal meningitis and with a CD4 count of 24 cells/μl. No viral load was available for this patient. Viral isolates were generated from both patients in a previous study using peripheral blood mononuclear cells (PBMC) cocultured with phytohemagglutinin (PHA)-activated donor PBMC (Cilliers et al., 2003). CM4-P1 was the first isolation reported in a previous publication (Trkola et al., 2001), which was later re-expanded using PHA-stimulated PBMC and IL-2 to generate high titer stocks for these experiments (CM4-P2). The gp160 encoding regions of both CM4-P1 and CM4-P2 were sequenced and shown to be more than 99% identical (Fig. 1A). CM4 was reported initially as an *env* subtype C by HMA (Bredell et al., 2002), but subsequent sequence analysis indicated that this virus was a complex recombinant of at least five different subtypes with multiple breakpoints in the *env* region (Papathanasopoulos et al., 2002). CM9 was subtype C throughout the genome (Papathanasopoulos et al., 2002). Ethical clearance for this study was obtained from the University of the Witwatersrand Committee for Research on Human Subjects (Medical).

Coreceptor assays on cell lines

Both viruses were tested for their ability to replicate in U87.CD4 cells transfected with either CCR5 or CXCR4 cells as previously described (Cilliers et al., 2003). In addition, U87.CD4 cell lines expressing CCR1 and GHOST.3 cell lines expressing Bob/GPR15, CCR1, CCR3 and CXCR6 were used to measure alternate coreceptor usage. Because all GHOST.3 cell lines express endogenous CXCR4, it was necessary to add 1.3 μM of AMD3100 to block this activity (Zhang and Moore, 1999). Viral replication was measured using an in-house p24 antigen assay (Trkola et al., 2001). Increasing p24 antigen levels in the presence of AMD3100 therefore indicated use of the transfected coreceptor. To measure CCR8-dependent replication, we used NP2/CD4 cells expressing CCR5, CXCR4 or CCR8 (Soda et al., 1999). For this, we used immunostaining as previously described (Clapham et al., 1992). HIV infected cells were fixed and stained with anti-HIV-1 p24 monoclonal antibodies (provided by the Centralised Facility for AIDS Reagents, UK) and anti-mouse Fab'2 fragments conjugated to β-galactosidase (Southern Biotechnology Associates, Inc.). Infected cells stained blue on addition of X-gal substrate (0.5 mg/ml 5-bromo-4-chloro-3-indolyl-*b*-galactopyranoside; Fisher Bioreagents Inc., 3 mM potassium ferrocyanide, 3 mM potassium ferricyanide and 1 mM magnesium chloride). Groups of blue-stained cells were regarded as foci of infection, and virus infectivity was estimated as FFU per milliliter (FFU/ml).

Δ32/Δ32 CCR5 PBMC cell preparation

Blood was drawn from two individuals identified with the Δ32/Δ32 CCR5 mutation and PBMC isolated and stimulated with PHA and IL-2 as previously described (Cilliers et al., 2003; Willey et al., 2003). AMD3100 (1 μM) was used to inhibit entry via CXCR4.

Entry inhibitor assays

Entry inhibitor assays were performed using PHA-stimulated PBMC from wt/wt (wild-type/wild-type) CCR5 and Δ32/Δ32 CCR5 donor cells. Cells were incubated with the specific inhibitor for 1 h before adding 1000 50% tissue culture infectious dose ($TCID_{50}$) per ml of virus evaluated on PBMC cultures. Assays were performed on 96 well plates using RPMI 1640, 10% fetal calf serum and IL-2 as previously described (Cilliers et al., 2003; Willey et al., 2003). Inhibitors tested included the anti-CCR5 Mab PRO140 (Progenics Pharmaceuticals, New York), chemokines RANTES (PeproTech Inc., Rocky Hill, NJ), I309, eotaxin, vMIP-I and CXCL16 (all from R&D Systems, Inc.) and the small molecule CXCR4 antagonist, AMD3100

(AnorMED Inc., Langley, Canada). Viral replication was measured by p24 antigen production or reverse transcriptase (RT) activity using an RT-enzyme-linked immunosorbent assay (CavidiTech, Uppsala, Sweden). Enfuvirtide (ENF) (American Peptide Company and Hoffmann-La Roche, Palo Alto, Ca) was used as previously described (Cilliers et al., 2004).

RNA extraction and amplification of the gp160 region

HIV-1 viral RNA was extracted from 200 μl of culture supernatant from different cell lines using the MagNA Pure LC Total Nucleic Acid Isolation Kit and automated MagNA Pure LC Instrument (Roche Diagnostics GmbH, Mannheim, Germany). A reverse transcription step was performed and the cDNA was used to amplify the gp160 region with primers as previously described (Gao et al., 1996). PCR products were purified using the High Pure PCR Product Purification kit (Roche Diagnostics GmbH, Mannheim, Germany). Sequencing reactions were performed in a MicroAmp 96 well optical reaction plate using the ABI PRISM Big dye Terminator v3.0 Cycle Sequencing kit and ABI PRISM 3100 automated sequencer. Sequence analysis was performed using the Sequencher version 4.0 software (Gene Codes Corporation). The ClustalX program was used for alignments.

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