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Virology 337 (2005) 384 – 398

VIROLOGY

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Uncoupling coreceptor usage of human immunodeficiency virus type 1 (HIV-1) from macrophage tropism reveals biological properties of CCR5-restricted HIV-1 isolates from patients with acquired immunodeficiency syndrome

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Received 23 March 2005; returned to author for revision 18 April 2005; accepted 27 April 2005
Available online 23 May 2005

Abstract

The mechanisms underlying the pathogenicity of CCR5-restricted (R5) human immunodeficiency virus type-1 (HIV-1) strains are incompletely understood. Acquisition or enhancement of macrophage (M)-tropism by R5 viruses contributes to R5 HIV-1 pathogenesis. In this study, we show that M-tropic R5 viruses isolated from individuals with acquired immunodeficiency syndrome (late R5 viruses) require lower levels of CD4/CCR5 expression for entry, have decreased sensitivity to inhibition by the entry inhibitors TAK-779 and T-20, and have increased sensitivity to neutralization by the Env MAb IgG1b12 compared with non-M-tropic R5 viruses isolated from asymptomatic, immunocompetent individuals (early R5 viruses). Augmenting CCR5 expression levels on monocyte-derived macrophages via retroviral transduction led to a complete or marginal restoration of M-tropism by early R5 viruses, depending on the viral strain. Thus, reduced CD4/CCR5 dependence is a phenotype of R5 HIV-1 associated with M-tropism and late stage infection, which may affect the efficacy of HIV-1 entry inhibitors.

Keywords: HIV-1; CCR5; Macrophage; Tropism; TAK-779; T-20; Inhibition; Neutralization; Sensitivity

Introduction

Progression of human immunodeficiency virus type 1 (HIV-1) infection from early, asymptomatic stages of disease to acquired immunodeficiency syndrome (AIDS) is associated with a switch in viral coreceptor specificity from CCR5 using (R5) viral strains to those able to use CXCR4 (X4) or both coreceptors (R5X4) in 40–50% of infected adults (Bjorndal et al., 1997; Connor et al., 1997; Karlsson et al., 1994; Koot et al., 1993; Tersmette et al., 1989) (reviewed in de Roda Husman and Schuitemaker, 1998). However, X4 or R5X4 variants are absent in 50–60% of HIV-1-infected individuals who progress to AIDS (de Roda Husman et al., 1999; Jansson et al., 1996, 1999; Karlsson et al., 2004; Koning et al., 2003) (reviewed in Gorry et al., 2005). Therefore, the persistence of an exclusive R5 viral population in vivo is sufficient to cause

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doi:10.1016/j.virol.2005.04.034

immunodeficiency in the majority of HIV-1-infected individuals who progress to AIDS.

Whilst much effort has been directed towards understanding the molecular basis of pathogenicity of late-emerging X4 and R5X4 viruses (Glushakova et al., 1995, 1998; Picchio et al., 1998) (reviewed in de Roda Husman and Schuitemaker, 1998), the molecular mechanisms underlying the pathogenicity of R5 HIV-1 strains are incompletely understood (Gorry et al., 2005). R5 viruses are intrinsically cytopathic but exert pathogenic effects that are distinct from those of X4 or R5X4 viruses (Fais et al., 1999; Grivel and Margolis, 1999; Harouse et al., 1999). R5 HIV-1 strains isolated from patients with AIDS (hereafter referred to as late R5 viruses) have enhanced macrophage (M)-tropism (Li et al., 1999; Tuttle et al., 2002) and cause increased levels of CD4+ T cell death (Kwa et al., 2003) compared with R5 HIV-1 strains isolated from asymptomatic individuals (hereafter referred to as early R5 viruses). Late R5 viruses were shown to have increased in vivo cytopathicity in HIV-1-infected SCID-hu mice compared with early R5 viruses by one study (Scoggins et al., 2000), although this conclusion was not reached by other in vivo and ex vivo studies (Berkowitz et al., 1999; Kreisberg et al., 2001). Late R5 viruses have decreased sensitivity to inhibition by the β-chemokine RANTES (regulated on activation, normally T-cell-expressed and -secreted) compared with early R5 viruses (Jansson et al., 1999; Karlsson et al., 2004; Koning et al., 2003). Recent evidence suggests that decreased RANTES sensitivity is attributed to an increased flexibility of the R5 envelope glycoproteins (Env) that subsequently alters the mode and efficiency of CCR5 binding (Karlsson et al., 2004). Together, these findings provide evidence that late R5 viruses have intrinsic properties distinguishing them from early R5 viruses which may enhance their cytopathic effects, and that these properties are likely to be linked to Env conformations that enhance CD4 and/or CCR5 interactions.

The coreceptor specificity of primary HIV-1 isolates is frequently used to define HIV-1 tropism (reviewed in Kedzierska et al., 2003). For example, R5 viruses are often collectively grouped as M-tropic viral strains. However, several studies have failed to establish a strict correlation between CCR5 usage and M-tropism of HIV-1 (Cheng-Mayer et al., 1997; Cunningham et al., 2000; Dittmar et al., 1997; Hung et al., 1999). In fact, further studies have demonstrated the presence of non-M-tropic R5 viruses, which were replication competent in primary CD4+ T cells but which could not productively infect macrophages (Gorry et al., 2001; Li et al., 1999). Thus, while CCR5 may be the main coreceptor for HIV-1 entry in macrophages, not all R5 viruses are M-tropic. In addition, some highly M-tropic primary HIV-1 strains use CXCR4 for entry in macrophages and microglia (Ancuta et al., 2001; Gorry et al., 2001; Koning et al., 2001; Naif et al., 2002; Ohagen et al., 2003; Singh et al., 2001; Yi et al., 1998, 1999). Furthermore, the principal source of plasma virus in macaques infected with a simian–human immunodeficiency virus (SHIV) strain that uses CXCR4 exclusively for virus entry is the tissue macrophage (Igarashi et al., 2001, 2003). Therefore, the viral determinants that underlie HIV-1 tropism for macrophages are significantly more complex than the coreceptor specificity of the virus.

Macrophages express lower levels of CD4, CCR5, and CXCR4 on the cell surface compared with CD4+ T cells (Lewin et al., 1996; Ometto et al., 1999; Wang et al., 2002), and low levels of these receptors expressed on macaque macrophages can restrict infection of some non-M-tropic R5 HIV-1 and X4 simian immunodeficiency virus (SIV) strains (Bannert et al., 2000; Mori et al., 2000). Furthermore, the level of CCR5 expression and CCR5 density can determine the level of susceptibility of human macrophages to infection by M-tropic R5 HIV-1 strains (Fear et al., 1998; Kuhmann et al., 2000; Rana et al., 1997; Reynes et al., 2001; Tuttle et al., 1998). Undifferentiated monocytes, which are refractory to infection by R5 HIV-1 viruses, become susceptible to infection by M-tropic R5 viruses upon differentiation concomitant with an increase in CCR5 expression levels (Collman et al., 1989; Di Marzio et al., 1998; Eisert et al., 2001; Fear et al., 1998; Naif et al., 1998; Neil et al., 2001; Rich et al., 1992; Sonza et al., 1996; Tuttle et al., 1998). Although restrictions to replication of R5 viruses in monocytes have been identified both pre- and post-reverse transcription (Eisert et al., 2001; Neil et al., 2001; Sonza et al., 1996; Triques and Stevenson, 2004), the major barrier to productive infection of differentiated monocyte-derived macrophages (MDM) and cultured microglia by non-M-tropic R5 HIV-1 strains is prior to reverse transcription (Gorry et al., 2001; Li et al., 1999). However, studies with identical twins showed that host cell genetics have an effect on the level of productive infection of M-tropic R5 viruses in MDM that is not related to CCR5 expression levels, and is exerted between viral entry and reverse transcription (Cunningham et al., 2000; Naif et al., 1999). Thus, levels of CD4 and/or CCR5 expression as well as post-entry stages of the HIV-1 replication cycle may be bottlenecks for productive infection of macrophages by R5 viruses.

The present study sought to better understand the biological properties of late-emerging primary R5 viruses that are important for M-tropism and R5 HIV-1 pathogenesis. We demonstrate two distinct phenotypes of primary R5 viruses; late, M-tropic R5 viruses have reduced dependence on CD4/CCR5 levels for entry and have reduced sensitivity to entry inhibitors TAK-779 and T-20. In contrast, early, non-M-tropic R5 viruses require comparatively higher levels of CD4/CCR5 for entry and are highly sensitive to inhibition by TAK-779 and T-20. Thus, enhancing CD4/CCR5 interactions may be a means by which R5 viruses increase their virulence and may affect the efficacy of HIV-1 entry inhibitors.

Results

Coreceptor usage

HIV-1 isolates were characterized for their ability to use CCR5, CXCR4, or alternative coreceptors for entry (Table 1). The X4, R5, and R5X4 strains NL4-3, ADA, and 89.6, respectively, were used as controls. NL4-3 used CXCR4 and Apj; ADA used CCR3, CCR5, CCR8, CX3CR1, Strl33, Gpr15, Gpr1, and Apj; and 89.6 used CCR2b, CCR3, CCR5, CXCR4, and Apj as coreceptors for virus entry, as described in previous studies (Churchill et al., 2004; Gorry et al., 2001, 2002b; Lawson et al., 2004). Early and late viruses used CCR5 as the sole coreceptor for entry, except NB7 where additional minor usage of CCR3 was evident. None of the viruses used CCR5 for entry in the absence of CD4 (data not shown). Thus, none of the primary HIV-1 viruses were CD4-independent, and all used CCR5 as the principal coreceptor for virus entry.

Replication kinetics

We examined the capacity of the early and late R5 viruses to replicate in MDM, PBMC, and the JC53 cell line. ADA was used as a control and replicated to high levels in the three cell types (Figs. 1A–C). Late R5 viruses replicated to high levels in all cell types, similar to ADA (Figs. 1G–I). Early R5 viruses replicated to high levels in JC53 cells (Fig. 1F), to moderately high levels in PBMC with delayed replication kinetics (Fig. 1E), but were unable to replicate or replicated poorly in MDM (Fig. 1D). Thus, the early R5 viruses have impaired replication capacity in MDM and can be classified as non-M-tropic R5 viruses as described previously (Gorry et al., 2001). JC53 cells have higher levels of CD4/CCR5 cell surface expression than PBMC (Platt et al., 1998, and data not shown), and PBMC have higher levels of CD4/CCR5 cell surface expression than MDM (Lewin et al., 1996; Ometto et al., 1999; Wang et al., 2002, and data not shown). Therefore, the results also raise the possibility that the diminished M-tropism by early R5 viruses may, at least in part, be attributed to an increased dependence on CD4 and/or CCR5 expression levels.

Effect of CD4 and CCR5 levels on infection by early and late R5 viruses

We next investigated whether late, M-tropic R5 viruses could utilize lower levels of CD4 and/or CCR5 for entry than early, non-M-tropic R5 viruses. Cf2-Luc cells were cotransfected with increasing amounts of CD4- and CCR5-expressing plasmid to create 16 populations of cells transiently expressing different amounts of either receptor, as described in previous studies (Gorry et al., 2001, 2002a). A linear relationship was found between the levels of CD4 and CCR5 expression and the amount of CD4- and CCR5-expressing plasmid used for transfection (Fig. 2), similar to previous studies (Gorry et al., 2001, 2002a; Martin et al., 2001; Shieh et al., 2000). In the 16 populations, the mean fluorescence intensity of CD4 remained constant when CCR5 levels were varied (Fig. 2A), and the mean fluorescence intensity of CCR5 remained constant when CD4 levels were varied (Fig. 2B). The ability of early and late R5 viruses to infect the 16-cell populations was examined (Fig. 3). The M-tropic ADA virus was used as a control. The infectivity of ADA, early, and late R5 viruses was equivalent when high levels of both CD4 and CCR5 were expressed. Late R5 viruses and ADA could infect cells expressing low levels of CD4 or CCR5 (Figs. 3A and C). However, the infectivity of late R5 viruses and ADA mediated by low levels of CD4 or CCR5 was generally dependent on the presence of medium or high

Table 1
Coreceptor usage by primary and reference HIV-1 isolates

	Coreceptor usage										
	CD4 only	CCR2b	CCR3	CCR5	CCR8	CXCR4	CX3CR1	Gpr1	Gpr15	Strl33	Apj
Controls											
NL4-3	–	–	–	–	–	+++	–	–	–	–	+
ADA	–	–	++	+++	+	–	+/–	+/–	+	+	–
89.6	–	+	++	+++	–	+++	–	–	–	–	+
Early R5 viruses											
NB23	–	–	–	+++	–	–	–	–	–	–	–
NB24	–	–	–	+++	–	–	–	–	–	–	–
NB25	–	–	–	+++	–	–	–	–	–	–	–
NB27	–	–	–	+++	–	–	–	–	–	–	–
Late R5 viruses											
NB2	–	–	–	+++	–	–	–	–	–	–	–
NB6	–	–	–	+++	–	–	–	–	–	–	–
NB7	–	–	+	+++	–	–	–	–	–	–	–
NB8	–	–	–	+++	–	–	–	–	–	–	–

Coreceptor usage of HIV-1 was determined using Cf2-Luc cells as previously described (Churchill et al., 2004; Gorry et al., 2001, 2002b; Lawson et al., 2004). Entry levels were scored as +++ (> 50,000 luciferase activity units), ++ (between 30,000 and 50,000 luciferase activity units), + (between 10,000 and 30,000 luciferase activity units), +/– (between 5,000 and 10,000 luciferase activity units), or – (< 5,000 luciferase activity units) as previously described (Gorry et al., 2001).

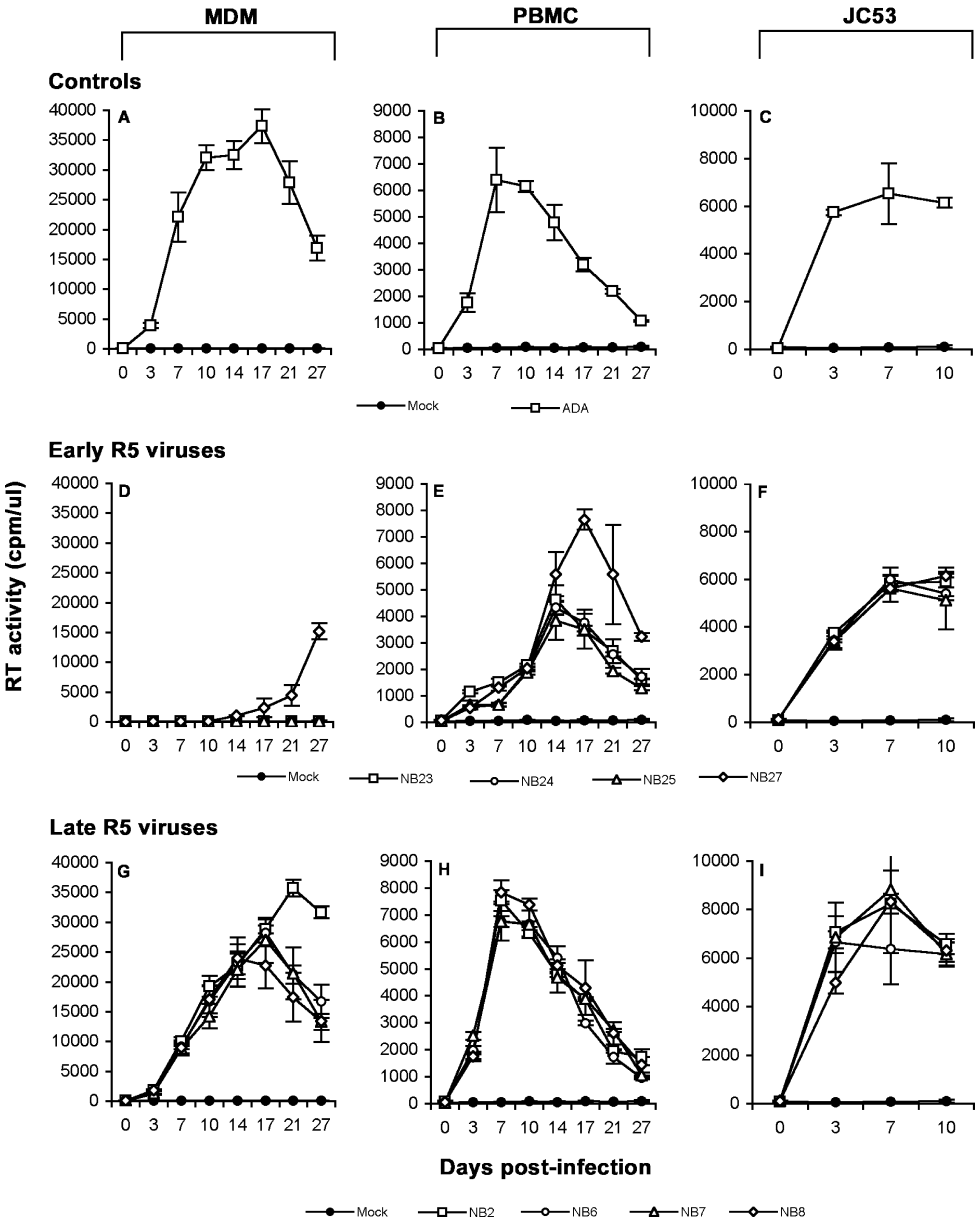

Fig. 1. Replication kinetics. MDM, PBMC, or JC53 cells were infected with equivalent amounts of each virus as described in Materials and methods, and cultured for 27 (MDM, PBMC) or 10 days (JC53). HIV-1 production in culture supernatants was measured by RT assays. Data are expressed as means from duplicate infections. Error bars represent standard deviations. Results are representative of three independent experiments. For experiments using MDM and PBMC, each independent experiment was performed using cells obtained from different donors.

levels of one receptor, consistent with previous studies of M-tropic R5 viruses (Kuhmann et al., 2000; Platt et al., 1998). In contrast, early R5 viruses required high levels of both CD4 and CCR5 expression for infection (Fig. 3B). Therefore, late M-tropic R5 viruses have reduced dependence on both CD4 and CCR5 levels for infection compared to early, non-M-tropic R5 viruses.

Transduction of MDM with retroviral vectors to modulate CD4/CCR5 expression

MDM were transduced with pSIvec1ΔenvhuCD4 to overexpress CD4 or with pSIvec1ΔenvhuCCR5 to overexpress CCR5. Control cultures were transduced with pSIvec1ΔenvGFP to express GFP or mock transduced with pSIvec1ΔenvEmpty. Thirty-six percent of MDM were effectively transduced, as determined by GFP expression (Fig. 4A). Mock-transduced MDM expressed low levels of CD4 on 84% of cells whereas CCR5 was expressed at low levels on only 13% of cells, and 12% of mock-transduced MDM were dually positive for both CD4 and CCR5 (Fig. 4B). Similar results were obtained using untransduced MDM and CD4-transduced MDM (data not shown). Thus, transduction of MDM with CD4-expressing vectors did not result in a further increase in CD4 expression levels. Transduction of MDM with CCR5-expressing vectors

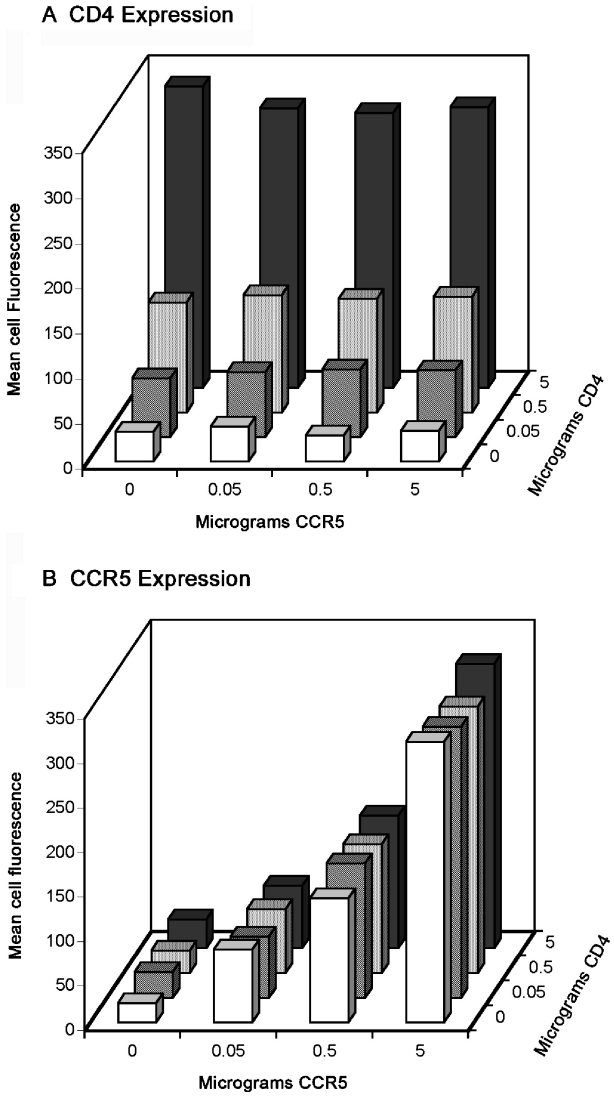

Fig. 2. CD4 and CCR5 expression in transfected Cf2-Luc cells. Cf2-Luc cells were cotransfected with 0, 0.05, 0.5, or 5.0 μg of CD4-expressing plasmid and 0, 0.05, 0.5, or 5.0 μg of CCR5-expressing plasmid to create 16 populations of cells which were analyzed for surface expression of CD4 (A) or CCR5 (B) by flow cytometry. Results are representative of two independent experiments.

resulted in an approximately 4-fold increase in the percentage of MDM staining positive for CCR5 and an approximately 2-fold increase in mean fluorescence intensity of CCR5 (Fig. 4B), resulting in 51% of CCR5-transduced MDM dually positive for both CD4 and CCR5. Thus, transduction of MDM with CCR5-expressing vectors resulted in an increase in CCR5 expression levels and an increase in the fraction of MDM that are potentially susceptible to HIV-1 entry.

Effect of CCR5 overexpression on HIV-1 replication in MDM

To determine whether the low level of CCR5 expression on MDM contributes to the diminished M-tropism by early R5 viruses, the replication kinetics of early and late R5 viruses was compared in MDM transduced with GFP or CCR5. Replication of early and late R5 viruses in GFP-transduced MDM (Figs. 5A and C) was similar to that in mock-transduced and -untransduced MDM (Figs. 1D and G, and data not shown). Transduction of MDM with CCR5 did not enhance replication of the late, M-tropic R5 viruses (Figs. 5C and D), but led to variable increases in replication of the early, non-M-tropic R5 viruses (Figs. 5A and B). NB27, which consistently replicated to low levels in GFP-transduced and -untransduced MDM, achieved levels of replication similar to late R5 viruses in CCR5-transduced MDM. In contrast, NB23, NB24, and NB25, which were consistently unable to replicate to detectable levels in GFP-transduced or -untransduced MDM, achieved only marginal levels of replication in CCR5-transduced MDM. These studies demonstrate that low CCR5 expression levels on MDM does not restrict replication of late, M-tropic R5 viruses, but is at least one bottleneck for productive infection by early, non-M-tropic R5 viruses.

Sensitivity of early and late R5 viruses to entry/fusion inhibitors

The preceding studies suggest that there may be differences in the efficiency of Env-CCR5 interactions between early and late R5 viruses, which may impact sensitivity to entry/fusion inhibitors. We therefore measured the sensitivity of early and late R5 viruses to inhibition by TAK-779 and T-20 as described in Materials and methods. The 50% inhibitory concentrations (IC_{50}) and IC_{90} are summarized in Table 2. A nonparametric Mann–Whitney *U* test showed that there was a significant increase in the IC_{50} and IC_{90} for TAK-779 ($P < 0.05$) and in the IC_{50} for T-20 ($P < 0.05$) against late R5 viruses compared with early R5 viruses. The differences in IC_{90} for T-20 between early and late R5 viruses were found not to be statistically significant ($P = 0.19$). Thus, late, M-tropic R5 viruses have reduced sensitivity to inhibition by TAK-779 and T-20 compared to early, non-M-tropic R5 viruses.

Sensitivity of early and late R5 viruses to antibody neutralization

We next measured the sensitivity of early and late R5 viruses to neutralization by MAbs IgG1b12, 2F5, and 2G12, and the polyclonal antibody HIV-Ig as described in Materials and methods. The IC_{50} and IC_{90} are summarized in Table 3. IgG1b12 neutralized the infectivity of all viruses, and a nonparametric Mann–Whitney *U* test showed that there was a significant decrease in the IC_{50} and IC_{90} for IgG1b12 against late R5 viruses compared with early R5 viruses ($P < 0.05$). 2F5 and 2G12 neutralized the infectivity of a subset of early and late R5 viruses and HIV-Ig neutralized the infectivity of all viruses, but the differences in IC_{50} and IC_{90} for 2F5, 2G12, and HIV-Ig between early

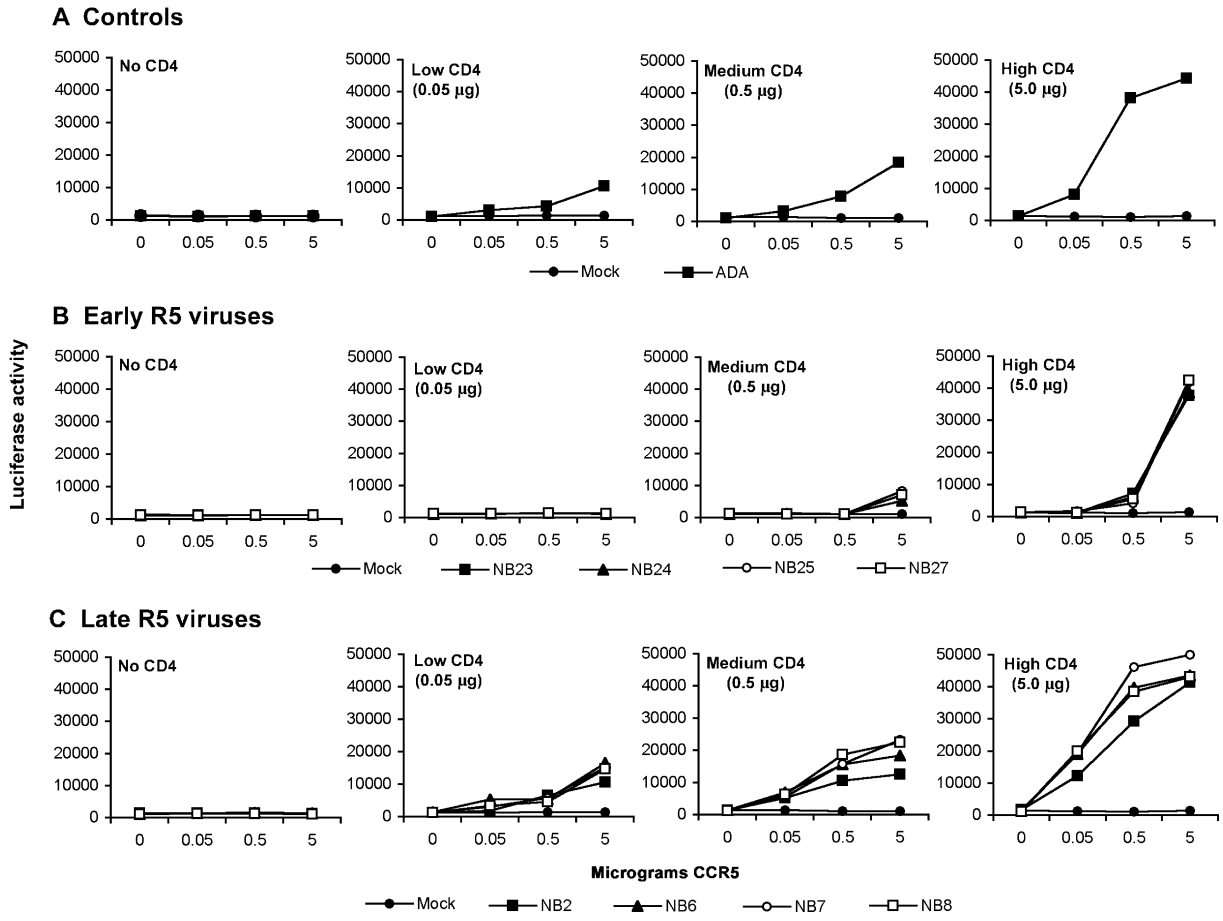

Fig. 3. Effect of CD4 and CCR5 levels on infection by early and late R5 viruses. Cf2-Luc cells were cotransfected with 0, 0.05, 0.5, or 5.0 μg of CD4-expressing plasmid and 0, 0.05, 0.5, or 5.0 μg of CCR5-expressing plasmid to create 16 populations of cells expressing either no, low, medium, or high levels of CD4 together with no, low, medium, or high levels of CCR5, as described previously (Gorry et al., 2001, 2002a; Martin et al., 2001; Shieh et al., 2000). Each cell population was infected with equivalent amounts of ADA (A), early R5 viruses (B), or late R5 viruses (C) as described in Materials and methods. Cell lysates were prepared at 48 h post-infection and assayed for luciferase activity. Results are representative of three independent experiments.

and late R5 viruses were found not to be statistically significant ($P > 0.08$). Thus, late, M-tropic R5 viruses displayed increased sensitivity to neutralization by IgG1b12 compared to early, non-M-tropic R5 viruses.

Discussion

In this study, we used a panel of early and late R5 HIV-1 viruses to investigate phenotypic characteristics that were associated with M-tropism and R5 HIV-1 pathogenesis. The early and late R5 viruses used in this study fell into two distinct phenotypes: late, M-tropic R5 viruses have reduced dependence on CD4/CCR5 levels for entry; whereas early, non-M-tropic R5 viruses require comparatively higher levels of CD4/CCR5 for entry. Limiting levels of CCR5 expression on MDM was at least partially responsible for the diminished M-tropism by early R5 viruses. Therefore, decreasing the dependence on CD4/CCR5 levels for entry may be one mechanism by which R5 viruses acquire or enhance tropism for macrophages. The phenotypic differences observed between early non-M-tropic and late M-tropic R5 viruses are likely to involve changes in the Env glycoproteins, which enhance Env-CCR5 interactions. This interpretation is supported by previous studies, which showed that late R5 viruses have decreased sensitivity to inhibition by the β-chemokine RANTES compared to early R5 viruses (Jansson et al., 1996, 1999; Koning et al., 2003), and by a more recent study which linked decreased RANTES sensitivity by late R5 viruses to alterations in the mode and efficiency of CCR5 usage by gp120 (Karlsson et al., 2004). In the present study, we further these findings by showing that a panel of late M-tropic R5 viruses has decreased sensitivity to the entry inhibitor TAK-779 and to the fusion inhibitor T-20 compared to a panel of early non-M-tropic R5 viruses.

The pathogenesis of R5 HIV-1 is poorly understood. Results of this study and those of other investigators suggest that R5 HIV-1 strains may evolve during infection toward more efficient CCR5 usage (Jansson et al., 1996, 1999; Karlsson et al., 2004; Koning et al., 2003) (reviewed in Gorry et al., 2005), although further studies of sequential virus isolates are required to determine the temporal nature of this evolution. While we have shown that more efficient

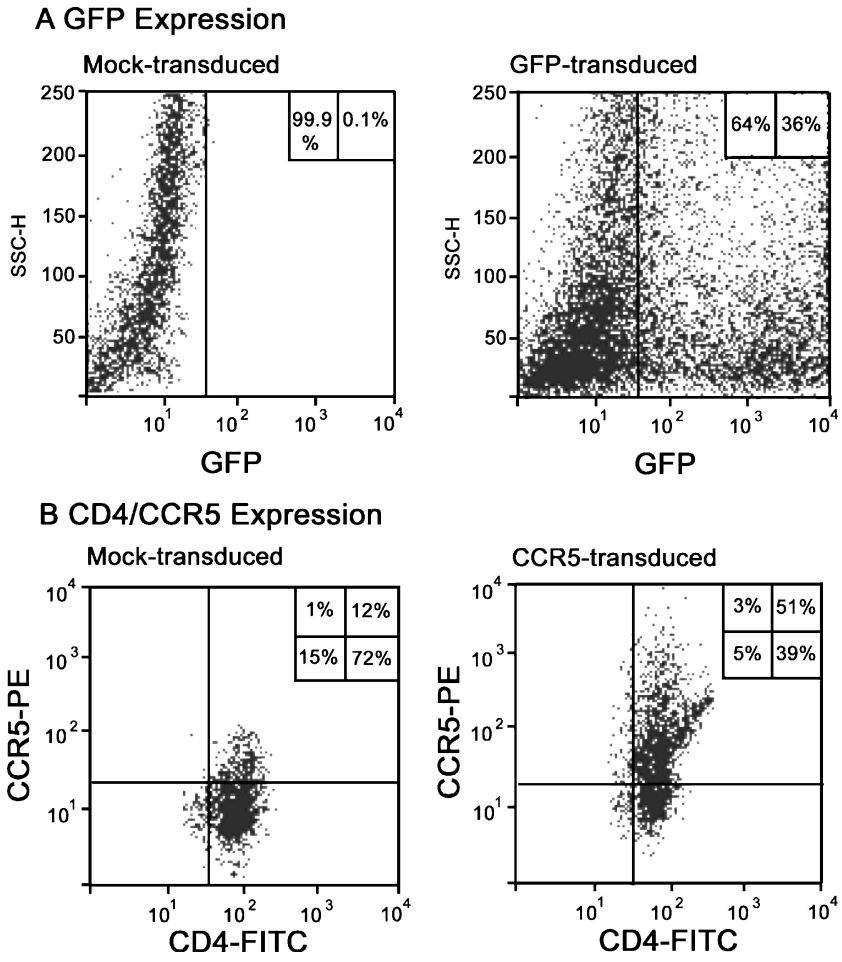

Fig. 4. Expression of GFP, CD4, and CCR5 in transduced MDM. MDM were mock-transduced with pSIvec1ΔEnvEmpty, or transduced with pSIvec1ΔEnvGFP or pSIvec1ΔEnvhuCCR5 to express GFP or CCR5, respectively, as described in Materials and methods. MDM were analyzed by flow cytometry for the expression of GFP (A), or for the expression of CD4 and CCR5 after staining with PE-conjugated anti-human CCR5 and FITC-conjugated anti-human CD4 antibodies (B). Values shown in the boxed areas represent the percentage of cells in the respective region of the scatter plot. Results are representative of two independent experiments using cells obtained from different donors.

CCR5 usage contributes to M-tropism of HIV-1, in a broader sense increased CCR5 usage by late R5 viruses may also contribute to the CD4+ T cell loss observed in 50–60% of patients who progress to AIDS while harboring R5 viral variants. This hypothesis is consistent with previous in vitro and in vivo studies that showed increased replicative capacity (Blaak et al., 1998; de Roda Husman et al., 1999; van 't Wout et al., 1998) and T cell cytopathicity (Kwa et al., 2003; Scoggins et al., 2000) by late R5 viruses compared to early R5 viruses, and consistent also with a previous study that demonstrated increased apoptosis of bystander T cells by R5 HIV-1 with increased gp120-receptor affinity and increased coreceptor binding site exposure (Holm et al., 2004). However, results of other in vivo and in vitro studies suggest that the pathogenicity of R5 strains may also be related to cytokine induction of increased CCR5 expression (Blaak et al., 2000; Choudhary et al., 2005). Together, the results of the present study and those of other investigators suggest that enhanced virus–receptor interactions and augmented immune activation are likely to be significant in the pathogenesis of R5 HIV-1 infection.

Late R5 viruses were found to be less sensitive to inhibition by TAK-779 and T-20 compared to early R5 viruses. Because late R5 viruses require lower levels of CCR5 for entry compared to early R5 viruses, and since late R5 viruses are more readily neutralized by the conformation-dependent MAb IgG1b12 than early R5 viruses, one interpretation of these findings is that an increase in CCR5 affinity by late R5 Envs may impede the efficacy of both entry inhibitors. This hypothesis is supported by results of a number of previous studies that have demonstrated the following: (i) reduced sensitivity of a neurovirulent R5 virus with increased Env-CCR5 affinity to inhibition by TAK-779 and another CCR5 inhibitor, SCH-C (Gorry et al., 2002a); (ii) HIV-1 escape from the CCR5 inhibitor AD101 which resulted, at least in part, by an increase in the affinity of Env for CCR5 (Trkola et al., 2002); (iii) increased sensitivity of the HIV-1 YU2 strain to inhibition by TAK-779 and T-20 when mutations in Env which reduce CCR5 affinity were

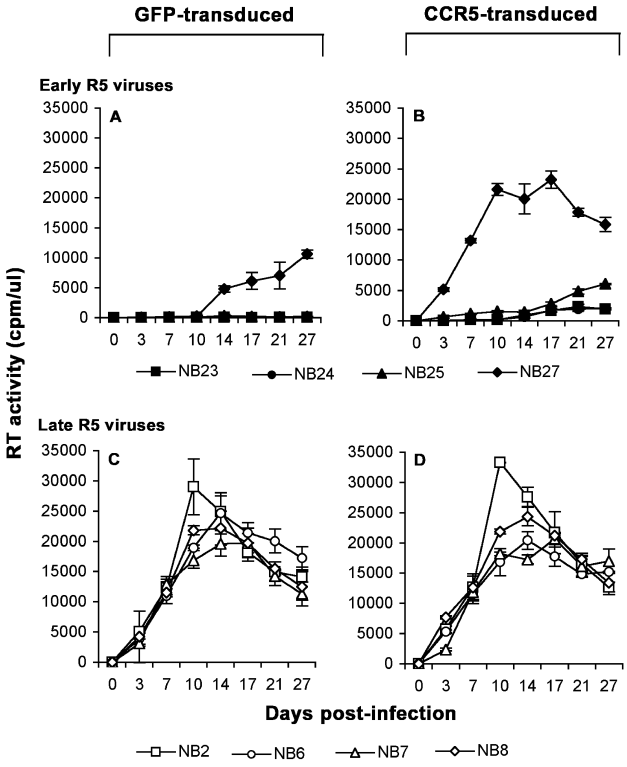

Fig. 5. Effect of high CCR5 expression on early and late R5 virus replication in MDM. MDM expressing high levels of CCR5 (B, D) or GFP as a control (A, C) were infected with equivalent amounts of each early R5 virus (A, B) or late R5 virus (C, D), as described in Materials and methods, and cultured for 27 days. HIV-1 production in culture supernatants was measured by RT assays. Data are expressed as means from duplicate infections. Error bars represent standard deviations. Results are representative of two independent experiments using cells obtained from different donors.

introduced (Reeves et al., 2002, 2004); and (iv) increased Env-mediated fusion kinetics by Envs with increased CCR5 affinity which decreased sensitivity to inhibition by T-20 (Reeves et al., 2002, 2004). Thus, an increase in the affinity of Env for CCR5 may reduce the sensitivity to entry inhibitors by at least two mechanisms: (i) by direct competition between small molecule inhibitors of CCR5 for CCR5 binding, and (ii) by increasing the rate of fusion limiting the opportunity for fusion inhibitors to be effective. Further studies of CCR5 affinity by Envs cloned from early and late R5 viruses are required to determine whether changes in Env-CCR5 affinity by primary R5 viruses affect the sensitivity to entry inhibitors.

Late R5 viruses were more sensitive than early R5 viruses to neutralization by IgG1b12, and there was a trend towards increased sensitivity of late R5 viruses to neutralization by 2F5, although this trend did not reach statistical significance. These results are strikingly similar to those of previous studies demonstrating that R5 Env clones from the neurovirulent UK1-br HIV-1 strain with increased CCR5 affinity were unusually sensitive to neutralization by IgG1b12, 2F5, and to the tetrameric CD4-IgG2 molecule

Table 2
IC_{50}s and IC_{90}s of HIV-1 entry inhibitors TAK-779 and T-20

Inhibitor		Early R5 viruses				Late R5 viruses			
		NB23	NB24	NB25	NB27	NB2	NB6	NB7	NB8
TAK-779	IC_{50}*	0.78	1.8	2.4	2.5	15	12	28	25
	IC_{90}*	33	40	43	40	68	52	>100	98
T-20	IC_{50}*	0.88	<0.1	0.6	2	7.5	10	6.5	13
	IC_{90}	17	2.6	10	>100	70	70	70	>100

IC_{50} and IC_{90} values of entry inhibitors TAK-779 (μM) and T-20 (μg/ml) for infection of Cf2-Luc cells expressing CD4 and CCR5 by early and late R5 viruses were calculated as described in Materials and methods.

* $P < 0.05$ for the difference in inhibitory concentrations between early and late R5 viruses by a nonparametric Mann–Whitney U test.

Table 3
IC_{50}s and IC_{90}s of Env MAbs and HIV-Ig

Antibody		Early R5 viruses				Late R5 viruses			
		NB23	NB24	NB25	NB27	NB2	NB6	NB7	NB8
IgG1b12	IC_{50}*	25	12	3.2	7	0.16	<0.1	2	2.8
	IC_{90}*	>100	68	18	>100	1.4	6.5	10	8.1
2F5	IC_{50}	R	15	12	R	2.6	0.9	6.1	R
	IC_{90}	R	>100	77	R	80	52	>100	R
2G12	IC_{50}	R	R	30	R	R	34	R	R
	IC_{90}	R	R	>100	R	R	>100	R	R
HIV-Ig	IC_{50}	54	19	<10	62	46	74	160	200
	IC_{90}	2800	410	730	8500	1000	750	3500	3400

IC_{50} and IC_{90} values of Env MAbs and HIV-Ig (μg/ml) for infection of Cf2-Luc cells expressing CD4 and CCR5 by early and late R5 viruses were calculated as described in Materials and methods. R, resistant to neutralization.

* $P < 0.05$ for the difference in inhibitory concentrations between early and late R5 viruses by a nonparametric Mann–Whitney U test.

(Gorry et al., 2002a), and to previous studies demonstrating that a CD4-independent variant of HIV-1 ADA (ADA197N/K) was unusually sensitive to neutralization by IgG1b12, 2F5, and sCD4 but not by the anti-CCR5 MAb 2D7 (Kolchinsky et al., 2001). In the latter study, the increased neutralization sensitivity to MAbs and sCD4 was thought to result from an increase in the exposure of the CCR5 binding domain and its associated antibody epitopes. The results of the present study suggest that late R5 virus Envs may have structural features that resemble those in HIV-1 UK1-br and ADA197N/K. The association between M-tropism, CD4/CCR5 dependence, and neutralization sensitivity by R5 HIV-1 strains demonstrated here is also similar to that found in a previous study of SIV strains, which showed a correlation between viral growth in cultures of alveolar macrophages, decreased CD4 dependence, and increased sensitivity to antibody neutralization (Means et al., 2001). Late, M-tropic R5 viral variants that have increased sensitivity to antibody neutralization may be able to replicate and persist in vivo during late stages of HIV-1 infection when the humoral immune response is waning (reviewed in Gorry et al., 2005). This hypothesis is supported by recent evidence which suggests that neutralization-sensitive R5 HIV-1 strains may be preferentially transmitted heterosexually (Derdeyn et al., 2004). Reduced selection pressure from antibodies may allow the evolution of R5 variants with increased CD4/CCR5 interactions, similar to that which occurs in immune privileged anatomical sites such as the brain (Gorry et al., 2001, 2002a; Martin et al., 2001; Peters et al., 2004; Shieh et al., 2000; Strizki et al., 1996). This would be consistent with a long standing concept that the protection of HIV-1 from neutralizing antibodies carries the price of a less efficient interaction with it's entry receptors (Moore and Ho, 1995).

Although early R5 viruses could replicate efficiently in JC53 cells and PBMC and were highly infectious for Cf2-Luc cells when engineered to express high levels of CD4/CCR5, none were able to replicate efficiently in MDM, and only NB27 could replicate to levels comparable to late R5 viruses when CCR5 was overexpressed on MDM. In contrast, only marginal increases in levels of replication could be detected in MDM cultures infected with early R5 viruses NB23, NB24, or NB25 when CCR5 was overexpressed. The fact that NB27 was able to replicate to low levels in untransduced MDM suggests that this virus may exist in a conformation that renders it more responsive to increases in CCR5 expression levels for entry into MDM. Alternatively, it is possible that infection of MDM may select for replication of a minor variant in the NB27 viral quasi-species which is enhanced by increasing CCR5 expression. The reasons underlying the discrepant infectivities of early R5 viruses NB23, NB24, and NB25 for infection of CD4/CCR5-expressing Cf2-Luc cells and CCR5-expressing MDM are unclear. Further studies are required to determine why increasing CCR5 expression levels on MDM was insufficient to rescue M-tropism by these early R5 viruses, but possibilities include limiting CD4 levels (Bannert et al., 2000; Mori et al., 2000), further post-entry blocks; for example, those exerted prior to (Cunningham et al., 2000; Naif et al., 1999) or at reverse transcription (Eisert et al., 2001; Neil et al., 2001; Sonza et al., 1996; Triques and Stevenson, 2004), or differences in CCR5 conformation (Doms, 2000; Hill et al., 1998) and/or post-translational modifications such as sulfation (Farzan et al., 1999) or O-linked glycosylation (Carlsson et al., 1986; Farzan et al., 1999; Fukuda et al., 1986) that exist between macrophages and other susceptible cell types. Differences in the stoichiometry and physical relationship of CD4/CCR5 expressed on Cf2-Luc cells compared to MDM may also be a bottleneck for infection of MDM by R5 viruses (Kuhmann et al., 2000; Platt et al., 1998). Other cell-specific factors (Doms, 2000), as well as viral factors such as Nef, could also affect the level of M-tropism by R5 viruses (Balliet et al., 1994; Miller et al., 1994).

In conclusion, reduced CD4/CCR5 dependence is a phenotype of R5 HIV-1 associated with M-tropism, R5 HIV-1 pathogenesis, and reduced sensitivity to inhibition by T-20 and TAK-779. T-20 is currently used as an anti-HIV-1 therapeutic, and several CCR5 inhibitors are in clinical trials or preclinical development. Whether late R5 viral variants with reduced CD4/CCR5 dependence will impede therapy by T-20 or CCR5 inhibitors remains to be determined by clinical studies. However, these findings underscore the importance of inhibiting late emerging, M-tropic R5 HIV-1 variants in the design of antiretroviral strategies aimed at inhibiting HIV-1 entry.

Materials and methods

Virus isolates

HIV-1_{NL4-3} and HIV-$1_{89.6}$ virus stocks were produced by transfection of 293T cells with proviral plasmid DNA (Adachi et al., 1986; Collman et al., 1992) by the calcium phosphate method (Gorry et al., 1998). HIV-1_{ADA} virus stocks (Gendelman et al., 1988) were prepared from supernatants of infected PBMC as described previously (Gorry et al., 2001). A detailed characterization of the primary HIV-1 viruses used in this study including Env V1, V2, and V3 sequence and heteroduplex mobility assay analysis of viral diversity, and clinical characteristics of the subjects from whom they were isolated, is described in a previous study (Li et al., 1999). Briefly, early HIV-1 viruses NB23, NB24, and NB25 were isolated from PBMC of individuals with CDC category II disease (asymptomatic infection) with CD4 counts of >500 cells/μl. Early virus NB27 was isolated from PBMC of an individual with CDC category I disease (acute seroconversion) with CD4 count of >750 cells/μl. Late viruses NB2, NB6, NB7, and NB8 were isolated from PBMC of individuals with CDC category IV disease (AIDS) and CD4 counts of <50 cells/μl. Virus

stocks were quantified by reverse transcriptase (RT) assay using [^{33}P]dTTP incorporation as described previously (Gorry et al., 1998), filtered through 0.45 μm pore size filters and stored at −80 °C.

Cells

PBMC were purified from blood of healthy HIV-1-negative donors, stimulated with 5 μg/ml of phytohemagglutinin (PHA) (Sigma, St. Louis, MO) for 3 days, and cultured in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum (FCS), 100 μg/ml of penicillin and streptomycin, and 20 U/ml of interleukin-2 (IL-2) (Roche, Basel, Switzerland). MDM were purified from PBMC by plastic adherence and cultured for 5 days in RPMI 1640 medium supplemented with 10% (vol/vol) human AB+ serum, and 100 μg/ml of penicillin and streptomycin. Cf2-Luc cells (Etemad-Moghadam et al., 2000), derived from the Cf2th canine thymocyte cell line (Choe et al., 1996), stably express the luciferase gene under the control of the HIV-1 long terminal repeat and were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (vol/vol) FCS, 100 μg/ml of penicillin and streptomycin, and 0.7 mg/ml of G418 (Gibco BRL, Gaithersburg, MD). JC53 cells are derived from the HeLa cell line and stably express high levels of CD4 and CCR5 on the cell surface (Platt et al., 1998), and were cultured in DMEM supplemented with 10% (vol/vol) FCS, and 100 μg/ml of penicillin and streptomycin.

Coreceptor usage

Coreceptor usage by primary HIV-1 isolates was determined using Cf2-Luc cells expressing CD4 alone, or expressing CD4 together with CCR2b, CCR3, CCR5, CCR8, CXCR4, CX3CR1, Gpr1, Gpr15, Strl33, or Apj, as described previously (Churchill et al., 2004; Gorry et al., 2001, 2002b; Lawson et al., 2004). Briefly, Cf2-Luc cells were transfected with 10 μg of plasmid pcDNA3-CD4 and 20 μg of plasmid pcDNA3 containing HIV-1 coreceptor using the calcium phosphate method, and infected 48 h later by incubation with 1×10^6 ^{33}P cpm RT units of HIV-1 in the presence of 2 μg/ml of Polybrene (Sigma). After overnight infection, virus was removed and the cells were cultured for an additional 48 h prior to lysis in 200 μl of cell lysis buffer (Promega, Madison, WI). Cell lysates were cleared by centrifugation and assayed for luciferase activity (Promega) according to the manufacturer's protocol.

HIV-1 replication kinetics

Five hundred thousand PHA-activated PBMC were infected in 48-well tissue culture plates by incubation with 1×10^6 ^{33}P cpm RT units of virus supernatant in a volume of 250 μl for 3 h at 37 °C, as described previously (Churchill et al., 2004). Virus was then removed, and PBMC were washed 3 times with phosphate-buffered saline (PBS) and cultured in medium containing 20 U/ml of IL-2 for 27 days. Fifty percent medium changes were performed twice weekly, and supernatants were tested for HIV-1 replication by RT assays. MDM were isolated from PBMC by plastic adherence and allowed to mature for 5 days prior to infection, as described previously (Gorry et al., 2001). At approximately 90% confluence in 48-well tissue culture plates, virus equivalent to 1×10^6 ^{33}P cpm RT units in a volume of 250 μl was allowed to adsorb to the cell monolayers for 3 h at 37 °C. Virus was then removed, and cells were rinsed 3 times with PBS prior to addition of 500 μl of culture medium. Fifty percent medium changes were performed twice weekly for 27 days, and supernatants were tested for HIV-1 replication by RT assays. JC53 cells cultured in 48-well tissue culture plates to approximately 50% confluence were infected by incubation with 1×10^6 ^{33}P cpm RT units of virus supernatant in a volume of 250 μl for 3 h at 37 °C. Virus was then removed, and cells were rinsed 3 times with PBS prior to addition of 500 μl of culture medium. Fifty percent medium changes were performed twice weekly for 14 days, and supernatants were tested for HIV-1 replication by RT assays.

CD4/CCR5 dependence assay

Cf2-Luc cells were cotransfected with 0, 0.05, 0.5, or 5.0 μg of plasmid pcDNA3-CD4 and 0, 0.05, 0.5, or 5.0 μg of plasmid pcDNA3-CCR5 using the calcium phosphate method to create 16 populations of cells expressing either no, low, medium, or high levels of CD4 together with no, low, medium, or high levels of CCR5, as described previously (Gorry et al., 2001, 2002a; Martin et al., 2001; Shieh et al., 2000). The total amount of plasmid DNA used in each transfection was adjusted to 10 μg with empty pcDNA3 plasmid. Under these conditions the cotransfection efficiency of Cf2-Luc cells is typically 60–70%, as determined by coexpression of GFP (data not shown). Transfection of Cf2-Luc cells with pcDNA3-CD4 or pcDNA3-CCR5 in amounts increasing from 0.05 to 5.0 μg results in a linear increase in CD4 or CCR5 expression levels, respectively (Gorry et al., 2001, 2002a). Infection of Cf2-Luc target cells and measurement of virus infection was performed as described above.

Retroviral transduction of monocyte-derived macrophages

The envelope-deficient SHIV vectors pSIvec1-ΔenvEmpty, pSIvec1ΔenvGFP, pSIvec1ΔenvhuCD4, and pSIvec1ΔenvhuCCR5 used to transduce MDM with no protein, GFP, CD4, or CCR5, respectively, have been described previously (Bannert et al., 2000; Hofmann et al., 1999). Transducing viruses pseudotyped with vesicular stomatitis virus G protein were produced by co-transfection of 293T cells with the pSIvec1Δenv vector plus pHCMV-G

and a Rev-expressing plasmid at a ratio of 20:5:5. At 72 h post-transfection, cell supernatants containing transducing viruses were cleared by centrifugation, filtered through 0.45 μm pore size filters, quantified by RT assays, and stored at −80 °C. Five-day-old MDM at approximately 90% confluence in 48-well tissue culture plates were transduced by overnight infection with 2×10^6 ^{33}P RT units of virus in a volume of 250 μl containing 5 μg/ml of Polybrene (Sigma). Virus was removed and cells were rinsed twice with culture medium prior to culturing for an additional 48 h. Transduced MDM were infected with HIV-1 isolates as described above, or analyzed for the expression of GFP in cells or CD4 and CCR5 on the cell surface as described previously (Gorry et al., 2001).

Virus inhibition studies

The effect of the CCR5 antagonist TAK-779 (Baba et al., 1999) and fusion inhibitor T-20 on virus infectivity was assayed using Cf2-Luc cells expressing CD4 and CCR5 as target cells for infection. Briefly, 2×10^4 Cf2-Luc target cells cultured in 48-well tissue culture plates were incubated for 30 min with a range of concentrations of TAK-779 (0.01–100 μM) or T-20 (0.01–100 μg/ml) prior to infection with 1×10^6 ^{33}P cpm RT units of each HIV-1 isolate in a volume of 250 μl for 3 h at 37 °C. Under these conditions, Cf2-Luc target cells support equivalent levels of virus infection by the primary HIV-1 isolates in the absence of inhibitor (data not shown). Mock-infected cultures of inhibitor-treated cells were incubated with 250 μl culture medium instead of virus supernatant. The inoculum was then removed, and cells were rinsed 3 times with PBS prior to addition of 500 μl of culture medium. Virus infectivity was measured by assaying luciferase activity in cell lysates at 72 h post-infection. After subtracting background luciferase activity of mock-infected cultures, the amount of luciferase activity in the presence of an inhibitor was expressed as a percentage of the amount produced in control cultures containing no inhibitor. The percent inhibition was calculated by subtracting this number from 100. Inhibition curves were generated using XLfit software (IDBS, Surrey, UK), and the IC_{50} and IC_{90} values were calculated by 4-parameter nonlinear regression. The differences in inhibitory concentrations between early and late R5 viruses were analyzed by a nonparametric Mann–Whitney *U* test.

Neutralization assays

Human monoclonal antibodies (MAb) against HIV-1 gp120 (IgG1b12 and 2G12) and gp41 (2F5) and the polyclonal antibody HIV-Ig have been described previously (Burton et al., 1991, 1994; Muster et al., 1994; Trkola et al., 1995, 1996). Neutralization of virus infectivity in Cf2-Luc target cells expressing CD4 and CCR5 was assessed by incubation of virus for 2 h at 37 °C with a range of concentrations of each MAb (0.01–100 μg/ml) or HIV-Ig (10–10,000 μg/ml) prior to infection, as previously described (Gorry et al., 2002a; Trkola et al., 1995). Infection of Cf2-Luc target cells, measurement of virus infectivity, and percent neutralization were calculated as described above. Inhibition curves were generated using XLfit software (IDBS, Surrey, UK), and IC_{50} and IC_{90} values were calculated by 4-parameter nonlinear regression. The differences in inhibitory concentrations between early and late R5 viruses were analyzed by a nonparametric Mann–Whitney *U* test.

Acknowledgments

We thank J. Sodroski B. Etemad-Moghadam and N. Bannert for providing Cf2-Luc cells and SHIV vectors, D. Kabat for providing JC53 cells, and D. Gabuzda, J. Sodroski, R. Doms, and S. Peiper for CD4 and coreceptor plasmids. The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 gp41 monoclonal antibody (2F5) and HIV-1 gp120 monoclonal antibody (2G12) from H. Katinger; HIV-1 gp120 monoclonal antibody (IgG1b12) from D. Burton and C. Barbas; HIV-Ig from NABI and NHLBI; TAK-779; and T-20, fusion inhibitor from Roche.

This study was supported by grants from the Australian National Health and Medical Research Council (NHMRC) to P.R.G (251520) and S.R.L. (251651 and 145802), and by a grant from NIH/NIAID to P.R.G, S.M.C and S.R.L. (1-R21-AI054207-01-A1). P.R.G. is a recipient of an NHMRC R. Douglas Wright Biomedical Career Development Award. S.M.C and S.R.L. are recipients of NHMRC Research Fellowships.

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