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Repits, Johanna; Öberg, Monica; Esbjörnsson, Joakim; Medstrand, Patrik; Karlsson, Anders; Albert, Jan; Fenyö, Eva Maria; Jansson, Marianne

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+46 46-222 00 00

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Selection of human immunodeficiency virus type 1 R5 variants with augmented replicative capacity and reduced sensitivity to entry inhibitors during severe immunodeficiency

Johanna Repits,[1] Monica Öberg,[1] Joakim Esbjörnsson,[1,2] Patrik Medstrand,[2] Anders Karlsson,[3] Jan Albert,[4] Eva Maria Fenyö[1] and Marianne Jansson[1,4]

Correspondence
Marianne Jansson
marianne.jansson@med.lu.se

[1,2]Department of Laboratory Medicine, Division of Medical Microbiology[1] and Department of Experimental Medicine[2], Lund University, Sölvegatan 23, 223 62 Lund, Sweden

[3]Venhälsan, Department of Infectious Medicine, Karolinska University Hospital, South Hospital, Stockholm, Sweden

[4]Department of Virology, Swedish Institute for Infectious Disease Control, Karolinska Institute, Stockholm, Sweden

Early in human immunodeficiency virus 1 (HIV-1) infection CCR5-using (R5) viruses predominate. With disease progression, approximately 50 % of infected individuals develop viruses able to use CXCR4. In the present work, the evolution of the biological properties of HIV-1 was studied in patients who retain viruses with an R5 phenotype despite AIDS onset. A panel of primary R5 HIV-1 isolates sequentially obtained at an asymptomatic stage and after AIDS diagnosis was examined. The viruses were selected based on our previous observation that R5 variants with reduced sensitivity to RANTES inhibition may appear during disease progression. Biological properties of the early and late R5 viruses, including infectivity, replicative capacity, impact of cationic polymer and sensitivity to inhibition by the entry inhibitors T-20 and TAK-779, were evaluated. R5 viruses isolated after AIDS onset displayed elevated replicative capacity and infectivity, and did not benefit from cationic polymer assistance during infection. Late R5 isolates also exhibited reduced sensitivity to inhibition by T-20 and TAK-779, even though the included patients were naïve to treatment with entry inhibitors and the isolates had not acquired mutations within the gp41 HR1 region. In addition, $CD4^+$ T-cell counts at the time of R5 virus isolation correlated with infectivity, replicative capacity and sensitivity to inhibition by entry inhibitors. The results indicate that R5 HIV-1 variants with augmented replicative capacity and reduced sensitivity to entry inhibitors may be selected for during severe immunodeficiency. At a time when the clinical use of entry inhibitors is increasing, this observation could be of importance in the optimal design of such treatments.

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INTRODUCTION

Many factors, including virus variability, can contribute to the pathogenicity of human immunodeficiency virus type 1 (HIV-1) infection. The high mutation rate and rapid turnover of HIV-1 result in a population of distinct viral variants, in other words a quasispecies, within the infected individual. Antiretroviral drugs and pressure exerted by the immune system of the host are selective forces that can contribute to the emergence of new HIV-1 variants in the viral population (Albert *et al.*, 1990; Mansky, 2002; Mansky *et al.*, 2002; Richman *et al.*, 2003). It has also been suggested that variable expression of viral receptors on target cells serves to select certain HIV-1 variants (van Rij *et al.*, 2000). Upon infection of the target cells, the HIV-1 envelope glycoprotein gp120 interacts with two different cellular receptors. gp120 attaches both to CD4 and to a coreceptor, which ultimately leads to a gp41-mediated fusion of the viral and cell membranes.

The coreceptors utilized by HIV-1 include CCR5 and/or CXCR4, which are seven transmembrane chemokine receptors (Feng *et al.*, 1996; Deng *et al.*, 1996; Dragic *et al.*, 1996; Cheng-Mayer *et al.*, 1997; Berger *et al.*, 1999). Early in HIV-1 infection, viruses primarily use CCR5 as a coreceptor

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are DQ156998–DQ157007.

(R5 phenotype) for host-cell entry (van't Wout *et al.*, 1994). Whether this is the result of a selective advantage at the time of virus transmission or when infection is established in the new host is yet to be determined. However, approximately half of the individuals infected with HIV-1 develop viruses at a later stage with the ability to use CXCR4 either solely (monotropic) or in combination with other chemokine receptors (dual-/multitropic) (Bjorndal *et al.*, 1997). The appearance of viruses using CXCR4 is correlated with an increased virulence and more aggressive disease progression (Koot *et al.*, 1992; Karlsson *et al.*, 1994; Connor *et al.*, 1997; Fenyö, 2001). Nevertheless, individuals who do not switch viral phenotype, and thus maintain an R5 virus phenotype throughout the course of the disease, eventually develop AIDS (de Roda Husman *et al.*, 1999; Jansson *et al.*, 1999). The natural CCR5 ligands RANTES, MIP-1α and MIP-1β can inhibit the replication of R5 viruses, whereas viruses using CXCR4 are resistant to inhibition by these chemokines (Cocchi *et al.*, 1995; Jansson *et al.*, 1996). However, our previous studies of patients who maintain the R5 virus phenotype throughout the course of the disease showed that R5 virus variants with reduced sensitivity to inhibition by RANTES may appear after AIDS onset (Jansson *et al.*, 1996, 1999). Entry inhibitors target the initial binding step between HIV and the target cell, and accordingly inhibit the fusion of the cellular and viral membranes (LaBranche *et al.*, 2001; Moore & Doms, 2003). The recent appearance of multidrug-resistant HIV-1 variants has triggered the research and development of alternative antiretroviral agents, including entry inhibitors. Thus, the establishment of the baseline susceptibility to entry inhibitors of HIV-1 variants emerging during disease progression may be important in the optimal design of such treatments.

With this in mind, we investigated the sensitivity to the entry inhibitors T-20 and TAK-779 of R5 viruses from patients who maintain the R5 phenotype during the course of the disease but develop R5 virus variants with reduced RANTES sensitivity after AIDS onset. Furthermore, additional biological properties, such as the infectivity and replicative capacity of these R5 isolates, were analysed.

METHODS

Patients and virus isolates. On the basis of our previous finding that R5 viruses with reduced sensitivity to RANTES may appear after AIDS onset, a panel of primary HIV-1 R5 isolates (Table 1) from five patients was selected from a larger cohort of homo- and bisexual men attending the South Hospital, Stockholm, Sweden (Karlsson *et al.*, 1991, 1994; Jansson *et al.*, 1996, 1999). Three of these patients, patients G, I and R, received antiretroviral monotherapy, Zidovudin (AZT) or Didanosine (ddI), during their disease. The isolates were obtained sequentially at different stages of the disease, both during the asymptomatic stage and after AIDS diagnosis. Virus stocks were generated by passaging R5 isolates in PHA-stimulated peripheral blood mononuclear cells (PBMC, Boule, Stockholm, Sweden). The viral R5 phenotype was determined (Jansson *et al.*, 1999) by infection of the coreceptor indicator cell lines GHOST and U87, kindly provided by Dr Dan Littman, Skirball Institute, New York University. Isolates from patient R (6322 and 8004, see Table 1) displayed the ability to use both CCR5 and CCR3 in the indicator cell lines. However, since these isolates did not replicate in donor PBMC lacking CCR5 expression (homozygous for the Δ32 deletion in the CCR5 gene) (Samson *et al.*, 1996; Jansson *et al.*, 1999), we classified them as being of R5 phenotype.

Table 1. Patient clinical status, CD4 count at time of virus isolation and coreceptor use of primary HIV-1 isolates studied

Patient*	Isolate	CD4 count†	Clinical status	Coreceptor‡
G	1228	260	Asymptomatic	R5
	4481	5	AIDS	R5
H	624	290	Asymptomatic	R5
	3899	6	AIDS	R5
I	5013	140	Asymptomatic	R5
	8616	90	AIDS	R5
M	668	750	Asymptomatic	R5
	7363	20	AIDS	R5
R	6322	200	Asymptomatic	R3R5
	8004	9	AIDS	R3R5

*Jansson *et al.* (1999).
†$CD4^+$ T cells μl^{-1}.
‡Coreceptor use determined by infection of U87.CD4 and GHOST(3) coreceptor indicator cell lines expressing CCR2b, CCR3, CCR5, CXCR4, CXCR6 or BOB (Jansson *et al.*, 1999).

PBMC replication assay and $TCID_{50}$. Leukocyte-concentrated peripheral blood from healthy donors was obtained from the Blood Center at Lund University Hospital, and PBMC were separated by a Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) gradient and frozen at $-130\,^{\circ}C$. PBMC used in the experiments originated from five donors whose cells were determined to be susceptible to R5 virus infections. Two days prior to infection, PBMC were stimulated by the addition of 2·5 $\mu g\ ml^{-1}$ phytohaemagglutinin (PHA) to RPMI 1640 glutamax (Invitrogen) supplemented with 0·1 $\mu g\ ml^{-1}$ streptomycin (Invitrogen), 0·1 $U\ ml^{-1}$ penicillin (Invitrogen) and 10 % fetal calf serum (FCS) (Invitrogen). PBMC (10^5 cells) were infected in infection medium [complete RPMI medium supplemented with 10 $U\ ml^{-1}$ interleukin 2 (Amersham Pharmacia)] with or without 2 $\mu g\ ml^{-1}$ of the cationic polymer polybrene (Sigma-Aldrich). The inoculum virus was normalized to a concentration of functional viral reverse transcriptase (RT) of 0·33 ng RT ml^{-1}, as measured by the CAVIDI HS kit (Cavidi Tech AB, Uppsala, Sweden) according to the manufacturer's instructions. In brief, in this assay, the concentration of functional RT activity as a measure of infectious virions (Corrigan *et al.*, 1998; Malmsten *et al.*, 2003; Marozsan *et al.*, 2004) is determined by the antibody-detected incorporation of bromo-deoxyuridine triphosphate (BrdUMP) during the synthesis of a DNA strand from an immobilized template/primer construct, mediated by RT present in the sample. After overnight virus infection at $37\,^{\circ}C$, 5 % CO_2, excess inoculum virus was removed by washing cells with PBS and the addition of fresh infection medium. Supernatants were harvested on days 4 and 7, and the p24 antigen content was analysed by ELISA (BioMérieux). For the determination of $TCID_{50}$, PHA-stimulated PBMC from two donors (10^5 cells per well) were infected with RT- or $TCID_{50}$-normalized virus, starting at 15 ng RT ml^{-1} or 12 × $TCID_{50}$, and serially diluted in fivefold steps in five parallel wells. After 4 h incubation, the cells

were washed with PBS and fresh infection medium was added. On day 7, supernatants were harvested and analysed for p24 antigen content by ELISA (BioMérieux).

U87.CD4-CCR5 infection assay. U87.CD4-CCR5 cells (Bjorndal *et al.*, 1997) were cultured in complete DMEM medium (Invitrogen) supplemented by 10 % FCS, 0·1 μg streptomycin ml^{-1} and 0·1 U penicillin ml^{-1}. Cells were seeded (0·5 ml) in 48-well plates and incubated overnight, or until they reached 50–60 % confluence. Infection was performed as described previously (Shi *et al.*, 2002), with the exception that inoculum virus (8·5 ng RT ml^{-1}) was RT-normalized and serially diluted in fivefold steps. On day 5, the cells were fixed in a methanol/acetone (1 : 1) mixture and stained with haematoxylin (Merck) to visualize the syncytia (plaque formation). The number of p.f.u. per well was determined by microscopic analysis.

Entry inhibitor sensitivity assay. For the analysis of R5 virus sensitivity to entry inhibitors, PBMC from four donors were pooled and infected as described above, with the exception that RANTES (PeproTech EC Ltd, London, UK), T-20 and TAK-779 were added. Infections were performed with inoculum virus normalized both to RT concentration (0·33 ng RT ml^{-1}) and to $TCID_{50}$ (40 ×). T-20 fusion inhibitor (Wild *et al.*, 1994; Kilby *et al.*, 1998) from Roche and TAK-779 (Baba *et al.*, 1999; Dragic *et al.*, 2000; Takashima *et al.*, 2001) were obtained from the NIH Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. Inhibitors were serially diluted in threefold steps, starting at the absolute concentrations of 45 nM T-20, 330 nM TAK-779 and 77 nM RANTES, and simultaneously added to the cells and virus. Control cultures without entry inhibitor were infected in parallel. Infected PBMC were washed with PBS on day 1 and fresh inhibitors at concentrations corresponding to the set-up were added to the cultures. Supernatants were harvested on days 4 and 7, and p24 antigen content was analysed by ELISA (BioMérieux). The sensitivity to entry inhibitors was evaluated as IC_{50} and calculated from p24 antigen release in the control cultures.

Sequencing of the gp41 HR1 region. The gp41 HR1 region of the R5 isolates studied was sequenced directly by obtaining RNA from virus stocks using NucleoSpin columns (Macherey-Nagel) according to the manufacturer's protocol. RNA was then transcribed to cDNA by reverse transcriptase PCR using random hexaoligonucleotides, Superscript II and RNase out (Invitrogen). For amplification of the specific gp41 fragment, PCR with *Pfx* polymerase (Invitrogen), forward primer 5′-CTTGGGAGCAGCAGGAAGCACT-3′ and reverse primer 5′-GGTGAGTATCCCTGCCTAACTCT-3′ (Invitrogen), was used. The amplified DNA fragment was then purified with the QIAquick PCR purification kit (Qiagen) and sequenced using forward sequencing primer 5′-CAGCAGGAAGCACTATGGGCG-3′, reverse sequencing primer 5′-TATCCCTGCCTAACTCTATTCACTA-3′ and the BigDye sequencing kit (Applied Biosystems). Sequences were then separated and analysed using a 3100 Genetic Analyser Hitachi (Applied Biosystems). The gp41 HR1 env sequences from the ten R5 isolates, corresponding to amino acids 21–80 of the HXB2 sequence, were aligned to 20 subtype B and two subtype D reference sequences from the Los Alamos database (http://www.hiv.lanl.gov/) using BioEdit software. Neighbour-joining phylogenetic trees were constructed from 516 unambiguously aligned and gap-stripped nucleotides using the MEGA version 3.0 software and the Kimura substitution model.

Statistical analysis. For the calculation of statistics, Statistica version 7 software was used. The non-parametric Spearman's rank correlation was used for the analysis of correlations, whereas Wilcoxon's matched pairs test was used for comparing the viral properties of early and late R5 isolates.

RESULTS

Late HIV-1 R5 isolates with reduced RANTES sensitivity display elevated replicative capacity and enhanced infectivity

The evolution of the biological properties of primary HIV-1 R5 isolates was examined. Isolates were selected from patients who retained the R5 viral phenotype throughout the disease course and developed R5 virus variants with reduced RANTES sensitivity, as previously described by this group (Jansson *et al.*, 1996, 1999) and confirmed in the present study ($P=0·043$) (Fig. 1a). The replicative capacity of the early and late R5 isolates, obtained sequentially from five patients prior to and after AIDS onset, respectively, was compared. PHA-stimulated donor PBMC were infected with inoculum virus normalized according to RT levels. By comparison of p24 antigen production on day 4 in cultures of five different donor-PBMC infections, the late R5 isolates were found to replicate better than the early isolates ($P=0·043$) (Fig. 1b). The enhanced replicative capacity and the reduced RANTES sensitivity of the late isolates led us to examine whether these viruses also differed in their capacity to induce infection of target cells. Infectivity was analysed in a $TCID_{50}$ assay in which the inoculum virus was normalized in terms of RT concentration. As shown in Fig. 1(c), the R5 viruses isolated after AIDS diagnosis exhibited significantly higher infectivity ($P=0·043$) than the viruses obtained from the corresponding patient prior to AIDS. To study changes in viral infectivity using a target cell with defined expression of CD4 and CCR5, we infected the coreceptor indicator cell line U87.CD4-CCR5 (Bjorndal *et al.*, 1997). In agreement with the results of the $TCID_{50}$ assay, late R5 isolates displayed higher infectivity in U87.CD4-CCR5 cultures than the early isolates ($P=0·043$) (Fig. 1d). In summary, we noted that late HIV-1 R5 isolates, in parallel to reduced RANTES sensitivity, displayed an elevated capacity to replicate in PBMC, and had an enhanced infectivity in PBMC as well as in the coreceptor indicator cell line U87.CD4-CCR5 compared with the early R5 isolates.

Differences in replication between early and late R5 isolates are augmented in the absence of cationic polymer

To further investigate the underlying mechanisms of the observed changes of viral properties between the early and the late R5 isolates, we performed PBMC infection experiments in which the cationic polymer polybrene was withdrawn from the infection medium. Polybrene is a cationic polymer routinely used to support viral infection during *in vitro* experiments. The mechanism of action has been suggested to involve non-specific membrane changes that aid the binding of the virus to the target cell (Davis *et al.*, 2002). Differences between the replication of early and late R5 isolates, presented as p24 antigen release, were clearly more pronounced in the absence of polybrene (Fig. 2). Unexpectedly, the late R5 isolates replicated better when the

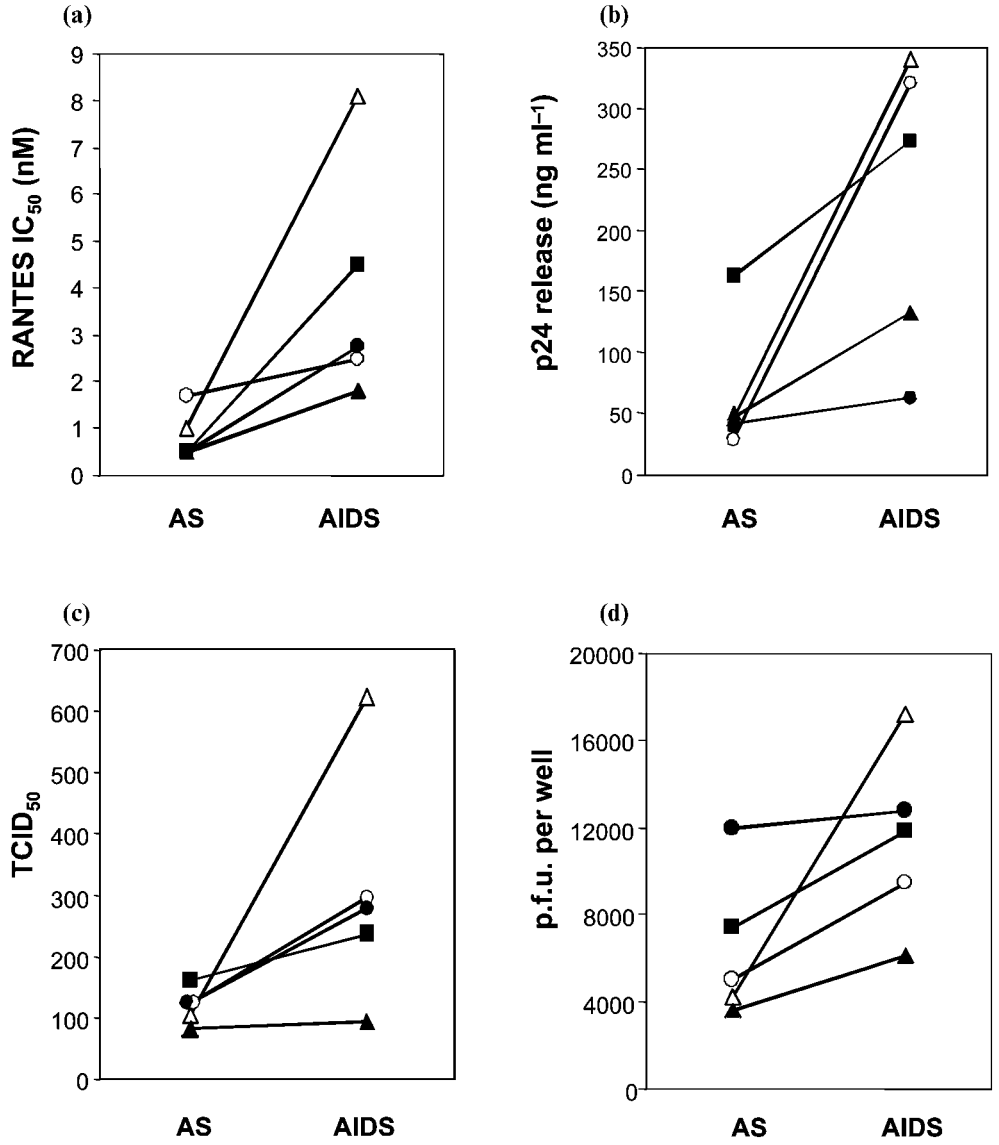

Fig. 1. Differences in RANTES sensitivity, replicative capacity and infectivity of R5 HIV-1 isolates obtained during asymptomatic (AS) infection and after AIDS onset. (a) RANTES sensitivity depicted as IC_{50} ($P=0{\cdot}043$, Wilcoxon matched pairs test). (b) Replication illustrated by mean p24 antigen release in infections of five different donor PBMC ($P=0{\cdot}043$, Wilcoxon matched pairs test). Infectivity measured by (c) PBMC $TCID_{50}$ and (d) p.f.u. in U87.CD4-CCR5 cells ($P=0{\cdot}043$ and 0·043, Wilcoxon matched pairs test, respectively). Presented data are the mean of triplicate wells from one representative experiment (out of two or more experiments performed). △, Patient G; ■, patient H; ▲, patient I; ○, patient M; ●, patient R.

cationic polymer was withdrawn. In addition, differences in infectivity between the early and late isolates, as assessed by $TCID_{50}$, were also more evident when polybrene was withdrawn from the medium (data not shown). This indicates that the physico-chemical membrane changes brought about by polybrene may in fact conceal the specific receptor interaction of R5 isolates with high replicative capacity, such as the late R5 isolates. The results suggest that early events in the replication cycle, such as receptor binding, may contribute to the observed changes in RANTES sensitivity, replication rate and infectivity.

Late R5 isolates with reduced RANTES sensitivity are more resistant to inhibition by the entry inhibitors T-20 and TAK-779

Since the polybrene experiments indicated that the altered binding of receptors might be involved in the changed viral properties observed, this prompted us to investigate whether

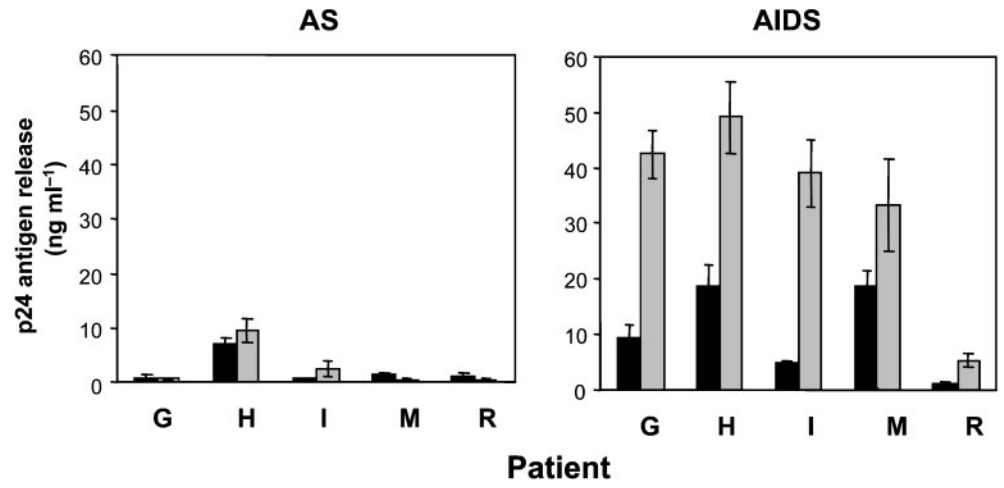

Fig. 2. R5 virus replication in PBMC in the presence (black bars) or absence (grey bars) of the cationic polymer polybrene. Results are presented as p24 antigen release in PBMC cultures infected with R5 isolates, sequentially obtained during the AS phase and after AIDS onset. Data are the mean of triplicate wells from one representative experiment (out of three).

R5 viruses less sensitive to RANTES inhibition also display altered sensitivity to other entry inhibitors. Early and late R5 isolates from the five patients, all naïve to entry-inhibitor treatment, were studied for sensitivity to the fusion inhibitor T-20 and the small-molecule CCR5 antagonist TAK-779 (LaBranche *et al.*, 2001; Moore & Doms, 2003), in parallel with RANTES (Fig. 1a). IC_{50} was determined in an inhibition assay employing RT-normalized inoculum virus, infection medium lacking polybrene and PBMC as the target cells. As shown in Fig. 3(a, b), the late R5 isolates were clearly less sensitive to inhibition by T-20 and TAK-779 than the early isolates ($P=0{\cdot}043$ and $0{\cdot}043$, respectively). The degree of RANTES sensitivity of R5 isolates also correlated with the level of T-20 and TAK-779 sensitivity ($P=0{\cdot}0072$, $r=0{\cdot}78$ and $P=0{\cdot}0057$, $r=0{\cdot}80$, respectively) (Fig. 3c and data not shown, respectively). As described above, we noted that late R5 isolates replicated better than early R5 isolates in PBMC cultures in which the inoculum viruses were normalized according to RT content. For comparison, inhibition assays with virus inoculum dose normalized according to $TCID_{50}$ were set up. In this case also we observed that the late R5 isolates were less sensitive to T-20 and TAK-779 ($P=0{\cdot}043$), regardless of the mode of normalization (Fig. 3d and data not shown, respectively). We also noted that none of the late R5 isolates was inhibited to 50 % by TAK-779 at the highest concentration tested. These results suggest that the R5 virus variants with reduced RANTES sensitivity that may emerge after AIDS onset also display reduced baseline sensitivity to the entry inhibitors T-20 and TAK-779. This, despite the fact that none of these patients or viruses had been exposed to T-20 or TAK-779.

Determinants for the natural emergence of R5 viruses with reduced sensitivity to entry inhibitors are not found within the gp41 HR1 region

Our previous analysis had excluded sequence variation within the gp120 V3 region as a determinant for phenotypic differences between early and late R5 isolates (Jansson *et al.*, 1999). To evaluate whether the emergence of R5 viruses with reduced sensitivity to entry inhibitors correlated with mutations within the gp41 heptad repeat 1 (HR1) region, previously reported to result in T-20 resistance (Rimsky *et al.*, 1998; Wei *et al.*, 2002), this region was sequenced. Phylogenetic analysis revealed that sequences clustered in a patient-specific manner, which argues against PCR contamination or sample confusion (data not shown). As shown in Table 2, none of the sequences displayed mutations in the HR1 region that have been associated with reduced sensitivity to entry inhibitors. In fact, the paired isolates from all patients were identical in the region, residues 36–45, known to generate most of the mutations previously linked to T-20 resistance. Thus, our results suggest that the determinants accounting for the natural emergence of R5 virus with reduced sensitivity to entry inhibitors lie outside the gp120 V3 and gp41 HR1 regions.

CD4 count at the time of isolation correlates to the biological properties of R5 virus

Next, we investigated whether the biological properties of R5 isolates was directly related to the degree of immunodeficiency during disease progression. As shown in Fig. 4(a), our analysis showed that within this group of patients there

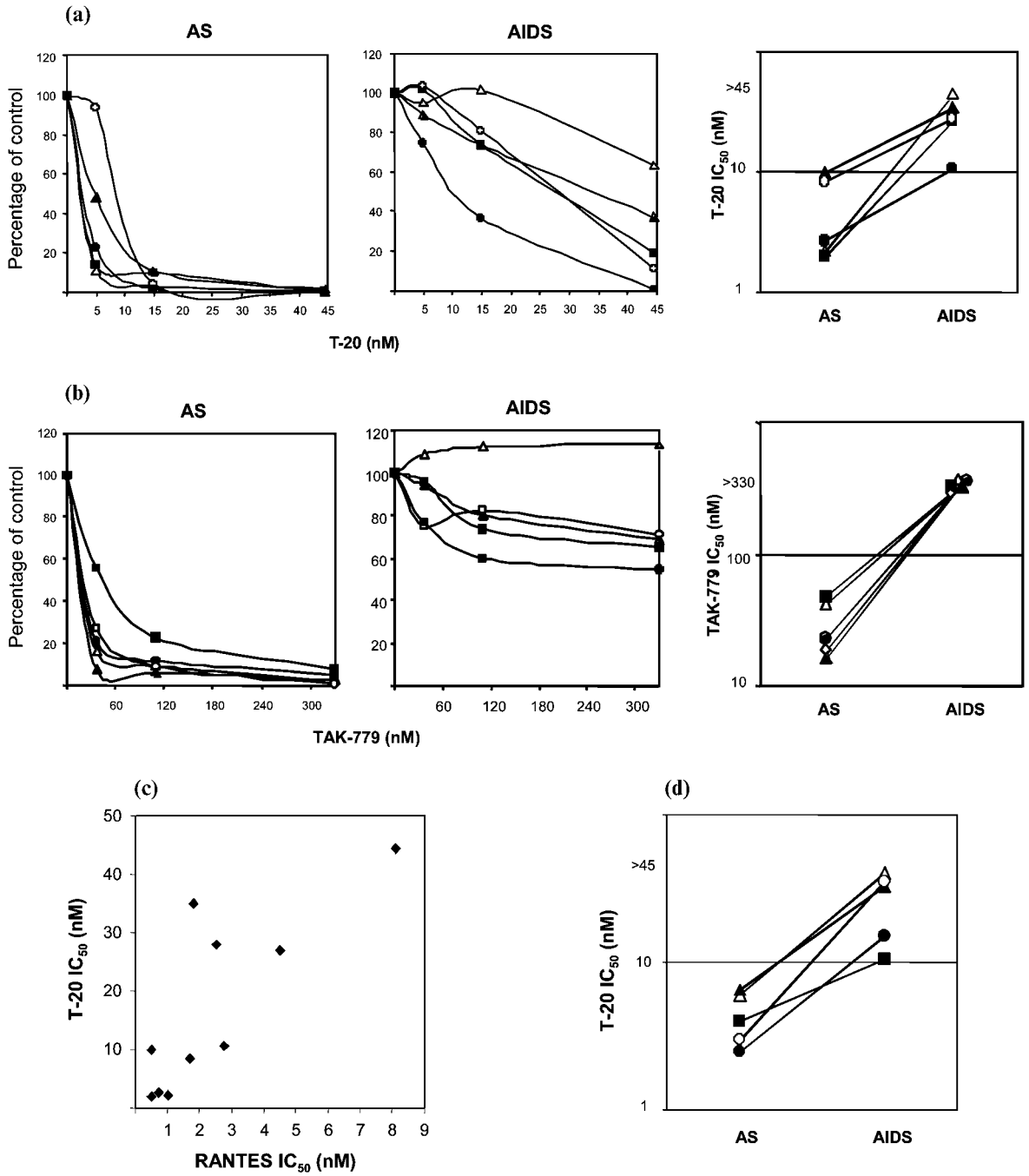

Fig. 3. Differences in sensitivity of early and late HIV-1 R5 isolates to inhibition by entry inhibitors. Reduced sensitivity of RT-normalized late R5 viruses, obtained after AIDS onset, to (a) T-20 and (b) TAK-779, compared with corresponding R5 isolates obtained at an AS stage ($P=0{\cdot}043$ and 0·043, Wilcoxon matched pairs test, respectively), and illustrated by inhibition curves and IC_{50}s. (c) Correlation between T-20 and RANTES IC_{50} in R5 virus inhibition ($P=0{\cdot}0072$, $r=0{\cdot}78$, Spearman's rank correlation). (d) T-20-mediated inhibition of $TCID_{50}$-normalized early and late R5 viruses ($P=0{\cdot}043$, Wilcoxon matched pairs test). The data presented are the mean of triplicate wells from one representative experiment (out of two or more experiments). For patient symbols, see legend to Fig. 1.

was a significant correlation between the number of $CD4^+$ T cells at the time of virus isolation and R5 virus replicative capacity in PBMC, assessed by p24 antigen release, ($P=0{\cdot}016$, $r=-0{\cdot}73$). We also found, as evident in Fig. 4(b–d), correlations between the CD4 count and R5 virus sensitivity to inhibition by entry inhibitors T-20, TAK-779 and RANTES ($P=0{\cdot}0038$, $r=-0{\cdot}82$; $P=0{\cdot}013$, $r=-0{\cdot}76$; $P=0{\cdot}0041$, $r=-0{\cdot}82$; respectively). These

Table 2. Comparison of gp41 HR1 region amino acid sequences of early and late R5 isolates

Patient	Isolate	Gp41 HR1 amino acid sequence*
		21 31 41 51 61 71
G	1228	AASLALTGQA RQLLSGIVQQ QNNLLRAIEA QQHLLQLTVW GIKQLQARVL AVERYLKDQQ
	4481	---I------ ---M------ ---------- ---------- ---------- ----------
H	624	AASMTLTVQA RLLLSGIVQQ QNNLLRAIEA QQHLLQLTVW GIKQLQARVL AVERYLRDQQ
	3899	---------- ---------- ---------- ---------- ---------- ----------
I	5013	AASITLTVQA RQLLSGIVQQ QNNLLRAIEA QQHLLQLTVW GIKQLQARVL AVERYLKDQQ
	8616	---------- ---------- ---------- ---------- ---------- ----------
M	668	AASITLTVQA RLLLSGIVQQ QNNLLRAIEA QQHLLQLTVW GIKQLQARVL AVERYLKDQQ
	7363	----A--A-- -Q-------- ---------- ---------- ---------- ----------
R	6322	AASMTLRVQA RLLLSGIVQQ QNNLLRAIEA QQHLLQLTVW GIKQLQARVL AVERYLKDQQ
	8004	---------- -Q-------- ---------- ---------- ---------- ----------

*Sequences aligned and numbered according to the HxB2 sequence. A dash in the late R5 virus sequence signifies an amino acid match with the early R5 virus sequence. Shaded sequences correspond to residues 36–45, which have been reported to contain mutations conferring T-20 resistance (Rimsky *et al.*, 1998; Wei *et al.*, 2002).

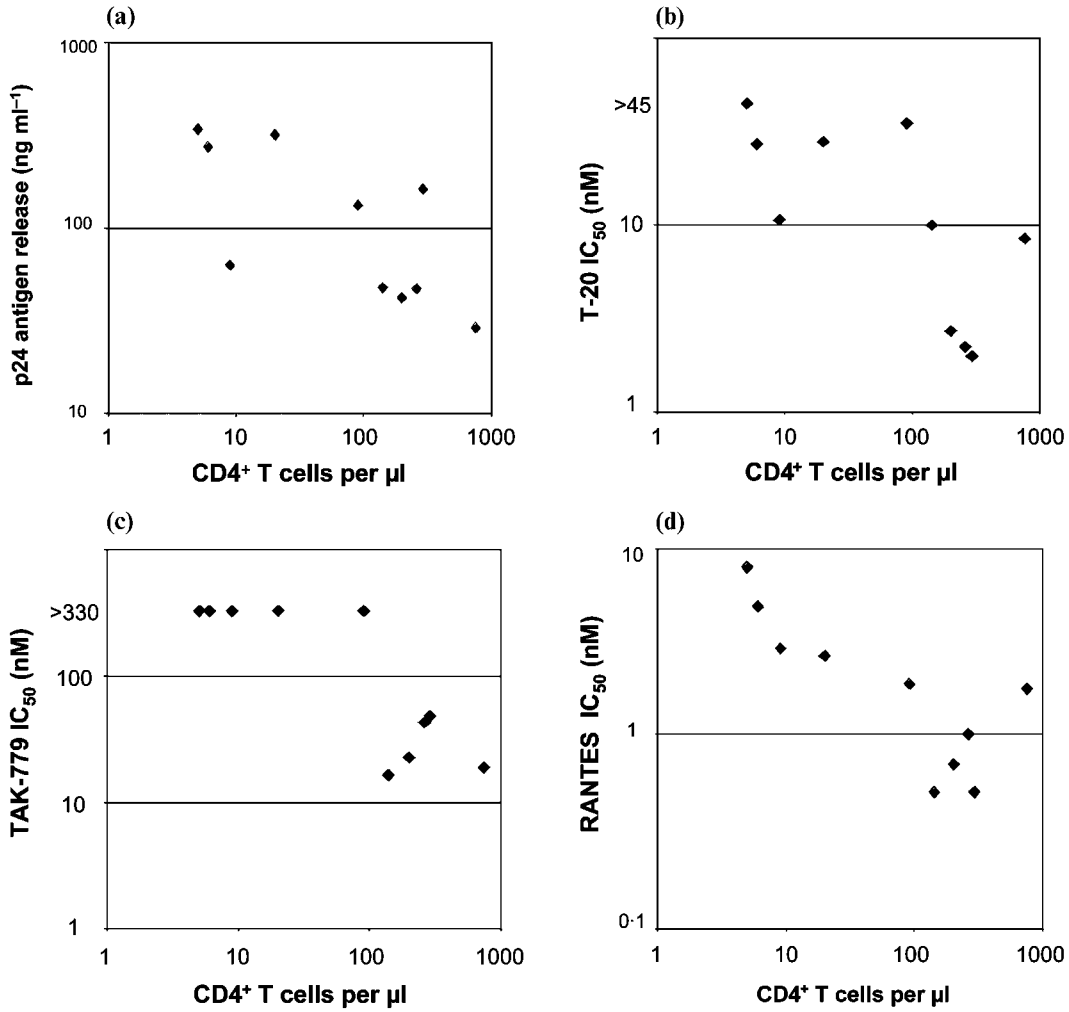

Fig. 4. Correlations between $CD4^+$ T cell counts at time of virus isolation and biological properties of early and late R5 HIV-1 isolates. CD4 count related to (a) replicative capacity measured as p24 antigen release in five different donor PBMC, sensitivity to inhibition by (b) T-20, (c) TAK-779 and (d) RANTES measured as IC_{50} values ($P=0{\cdot}016$, $r=-0{\cdot}73$; $P=0{\cdot}0038$, $r=-0{\cdot}82$; $P=0{\cdot}013$, $r=-0{\cdot}76$ and $P=0{\cdot}0041$, $r=-0{\cdot}82$, respectively, Spearman's rank correlation).

results suggest that, with declining numbers of $CD4^+$ T cells, R5 HIV-1 variants with augmented replicative capacity and reduced sensitivity to entry inhibitors may emerge.

DISCUSSION

The biological properties of sequentially isolated R5 HIV-1 from patients who maintain virus with R5 phenotype throughout the course of the disease, and who develop R5 virus variants resistant to inhibition by RANTES, were analysed. Our results show that R5 virus variants with reduced RANTES sensitivity also display reduced sensitivity to the entry inhibitors T-20 and TAK-779. Such viruses show augmented infectivity and replicative capacity, and these parameters correlate with decreasing numbers of $CD4^+$ T cells. We suggest that severe immunodeficiency selects for R5 virus variants with altered biological properties.

Both our work and that of others has demonstrated that also in patients who maintain the R5 viral phenotype throughout the disease course, viral evolution occurs *in vivo* (Jansson *et al.*, 1996, 1999; Koning *et al.*, 2003; Karlsson *et al.*, 2004). Our earlier findings have shown that R5 virus variants isolated after AIDS onset may display decreased sensitivity to RANTES inhibition (Jansson *et al.*, 1996, 1999). This observation has recently been confirmed by Koning *et al.* (2003), who found that RANTES-resistant R5 virus variants appeared as the disease progressed. These findings suggest an evolution of viral properties towards enhanced viral fitness with respect to the earliest events in the infection cycle, such as receptor binding. In the present study we corroborate this hypothesis by demonstrating that R5 isolates with reduced RANTES sensitivity also show enhanced infectivity and replicative capacity.

Interestingly, we recently noted that R5 virus variants with an altered mode of coreceptor use, in the form of a broadened ability to use CCR5/CXCR4 chimeric receptors, correlated with reduced RANTES sensitivity and decreasing numbers of $CD4^+$ T cells (Karlsson *et al.*, 2004). The assumption that the mode of receptor use is altered is also supported by the observation that late R5 viruses did not benefit from cationic polymer assistance. This was an unexpected observation, since polybrene has previously been shown to enhance retroviral adsorption rate (Davis *et al.*, 2002), and thus warrants further investigation. The mechanism of action of the cationic polymer polybrene has been suggested to involve the non-specific equalizing of charge differences between the glycocalyx on the target cell and the viral membrane (Davis *et al.*, 2002). Thus, late R5 isolates appear to have developed stronger, specific receptor binding.

Further support for the evolution of R5 variants with altered receptor binding-properties is provided by our finding that R5 viruses obtained after AIDS onset may be less sensitive to inhibition by the entry inhibitors TAK-779 and T-20. TAK-779, a small-molecule CCR5 antagonist binding to a pocket between CCR5 transmembrane helixes 1, 2, 3 and 7, has previously been shown to exert selective anti-HIV activity towards R5 viruses (Baba *et al.*, 1999; Dragic *et al.*, 2000; Takashima *et al.*, 2001). T-20, on the other hand, is a synthetic peptide corresponding to a gp41 segment that inhibits HIV of both R5 and X4 phenotypes by blocking the step preceding fusion of the viral and cellular membranes (Wild *et al.*, 1994; Kilby *et al.*, 1998). Thus, the development of R5 virus variants with reduced sensitivity to both CCR5 agonist and antagonist, RANTES and TAK-779, in addition to the fusion inhibitor T-20, suggests that these virus variants may have developed an altered ability to bind the HIV-1 receptors, by the modification of binding affinity, receptor binding site or fusion kinetics. Recently, it has been suggested by Reeves *et al.* (2002) that sensitivity to blockade by T-20 and TAK-779 correlates with R5 virus binding affinity. High CCR5 affinity results in more rapid fusion kinetics. In an experimental system, it has also been shown that *in vitro* passage of R5 virus in the presence of AD101, another small-molecule CCR5 antagonist, selects for a highly resistant escape mutant still dependent on CCR5 for host-cell entry but with increased CCR5 affinity (Trkola *et al.*, 2002). Accordingly, HIV-1 evolution is not restricted to a switch in coreceptors, but may also include modifications in the utilization of the coreceptor currently employed.

It is known that the expression levels of β-chemokines, being ligands of CCR5, are elevated in HIV-1-infected individuals (Clerici *et al.*, 1996; Ullum *et al.*, 1998). It is possible that CCR5 ligands may exert selection pressure for the development of R5 variants with improved binding properties due to down-modulation of CCR5. An altered cytokine milieu resulting in the reduced expression of CCR5 has also been reported to affect viral phenotype (Valentin *et al.*, 1998; Patterson *et al.*, 1999; Llano *et al.*, 2001). Alternatively, the lack of proper immune response during severe immunodeficiency may allow the evolution of HIV-1 variants with an altered biological phenotype. In line with this, coinfection with a mix of R5 and X4 simian/human immunodeficiency virus (SHIV) strains in a rhesus macaque model (Harouse *et al.*, 2003), suggested that X4 strains were preferentially suppressed in the immunocompetent host. On the other hand, since our results demonstrate that the number of $CD4^+$ T cells at time of isolation is associated with altered biological properties of R5 viruses, it is tempting to speculate that selective pressure acts upon the virus to evolve new properties in order to be able to infect the limited numbers of target cells. The evolution of certain virus variants following the selective loss of particular $CD4^+$ T-cell subsets has been reported (Blaak *et al.*, 2000), and reduced dependence on CCR5 and CD4 expression has also been suggested to be linked to R5 virus affinity and macrophage tropism (Gorry *et al.*, 2002).

In order to evaluate the infectivity, replicative capacity and sensitivity to entry inhibitors of different R5 variants, care was taken to minimize interference from viral binding properties. We chose to normalize the virus inoculum on the basis of functional RT, since RT activity has been shown

to correlate more closely with the number of infectious viral particles than does the quantity of p24 antigen (Corrigan *et al.*, 1998; Malmsten *et al.*, 2003; Marozsan *et al.*, 2004). This is because p24-capturing assays measure the presence of the p24 antigen (i.e. infectious, non-infectious and decaying viruses), whereas the RT assay measures the enzymic activity of the reverse transcriptase, which is rapidly lost outside an intact virus particle. Nevertheless, we chose to analyse sensitivity to entry inhibitors in two ways, by using both RT and $TCID_{50}$ normalization of inoculum virus. Both of these analyses revealed that late R5 viruses, less sensitive to RANTES, also displayed reduced sensitivity to the entry inhibitors T-20 and TAK-779. Altered envelope incorporation onto virions has recently been reported to influence both SIV and HIV infectivity (Yuste *et al.*, 2004; Bachrach *et al.*, 2005). Even though our analysis demonstrated that early and late R5 viruses differ in entry-inhibitor sensitivity when inoculum virus is normalized on the basis of $TCID_{50}$, we cannot exclude that variation in envelope incorporation may contribute to the observed differences in infectivity and replicative capacity. Thus, the quantification of envelope density on virions of early and late R5 isolates merits further investigation.

Treatment with T-20, also known as enfuvirtide, has revealed that resistant HIV-1 variants may emerge as the result of mutations within the HR1 region of gp41 (residues 36–45) (Rimsky *et al.*, 1998; Wei *et al.*, 2002; Greenberg *et al.*, 2004). The R5 isolates used in our study were all derived from patients who were naïve to T-20 treatment, and sequence analysis revealed that none of the early or late isolates had acquired any of the mutations within the gp41 HR1 region previously linked to T-20 resistance. However, another study on the characterization of baseline susceptibility to T-20 has also suggested that variation among primary HIV-1 isolates from patients naïve to entry-inhibitor treatment may exist without polymorphisms in the HR1 region of gp41 (Labrosse *et al.*, 2003). This is in line with our observations, yet our results reinforce that the variability in sensitivity to T-20 inhibition of R5 HIV-1 variants is associated with disease progression. In contrast to Labrosse *et al.* (2003), who did not investigate sequential isolates obtained before and after AIDS onset, we found that the sensitivity to RANTES inhibition of the R5 isolates studied correlated with sensitivity to T-20. Our previous analysis excluded sequence variation within the gp120 V3 region as a determinant for phenotypic differences between early and late R5 isolates (Jansson *et al.*, 1999). Taken together, these results suggest that determinants that account for the natural emergence of R5 variants of lower sensitivity to entry inhibitors have to be sought outside the gp120 V3 and gp41 HR1 regions.

In addition to the acquisition of basic knowledge regarding HIV-related pathogenesis, studies on the evolution of R5 HIV-1 variants with improved binding abilities that result in enhanced viral fitness may prove to be important for the identification of the mechanisms that determine baseline susceptibility to entry inhibitors, and so aid efforts to design optimal treatment strategies.

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