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Comparative studies on mucosal and intravenous transmission of simian immunodeficiency virus (SIVsm): evolution of coreceptor use varies with pathogenic outcome

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Coreceptor usage of isolates from 30 cynomolgus macaques infected intrarectally ($n=22$) or intravenously ($n=8$) with simian immunodeficiency virus of sooty mangabey origin (SIVsm) was evaluated in U87.CD4 and GHOST(3) cell lines. Based on progression rate, the animals were divided into progressors (18 animals), slow progressors (five animals) and long-term non-progressors (seven animals). There was no difference in how many or which coreceptors were used according to route of infection. All isolates but one used CCR5 for cell entry, and CCR5 was also the major coreceptor in 70 out of 105 isolates tested. In general, early isolates were multitropic, using CCR5, CXCR6 and/or gpr15. Interestingly, CXCR4-using viruses could be isolated on human peripheral blood mononuclear cells (PBMCs), but not on cynomolgus macaque PBMCs, suggesting that human PBMCs select for variants with CXCR4 use. Even though CXCR4-using SIV isolates have been reported rarely, we could recover CXCR4-using viruses from 13 monkeys. CXCR4 use either appeared early during the acute phase of infection and disappeared later or only appeared late in infection during immunodeficiency. Surprisingly, one late isolate from a progressor monkey did not use CCR5 at all and used the CXCR4 receptor with high efficiency. The ability to use many different receptors decreased over time in long-term non-progressor monkeys, whilst the majority of progressor monkeys showed broadening of coreceptor use, stable coreceptor use or fluctuation between the different coreceptor-usage patterns. The results indicate that, in the infected host, evolution of SIV coreceptor usage occurs, involving changes in the mode of coreceptor use.

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INTRODUCTION

Simian immunodeficiency virus (SIV) infection in macaques has been used extensively for modelling of human immunodeficiency virus (HIV) infection in humans. There are many similarities between the two systems: clinical signs of immunodeficiency are comparable (Kestler *et al.*, 1990; Letvin *et al.*, 1985) and the viruses enter cells through interaction of the viral envelope with CD4 and a coreceptor on the cell surface. The two major coreceptors used by HIV-1 are CCR5 and CXCR4 (Deng *et al.*, 1996; Dragic *et al.*, 1996; Feng *et al.*, 1996). Viruses using the CCR5 coreceptor (R5 phenotype) predominate early in asymptomatic HIV-1 infection, whilst CXCR4-using viruses (X4 phenotype) can be isolated in approximately half of patients that progress to AIDS (Björndal *et al.*, 1997; Karlsson *et al.*, 1994). Thus, CXCR4-using HIV-1 isolates are regarded as more pathogenic. CXCR4-using viruses are often characterized by dual tropism (R5X4) or multitropism and may use CCR5, CCR3 and/or CCR2b in combination with CXCR4. CCR5 is also the major coreceptor for SIV (Chen *et al.*, 1997; Edinger *et al.*, 1997; Marcon *et al.*, 1997), whereas CXCR4 use is rare and has been shown with virus isolated and grown on human peripheral blood mononuclear cells (hPBMCs) (Owen *et al.*, 2000; Schols & de Clercq, 1998; Vödrös *et al.*, 2001a). Instead, SIV isolates often use CXCR6 and the orphan receptor gpr15/BOB (Alkhatib *et al.*, 1997; Deng *et al.*, 1997; Farzan *et al.*, 1997). Use of CXCR6 and gpr15 by HIV-1 is much less frequent. Furthermore, both HIV and SIV have been shown to use a wide set of alternative coreceptors, including CCR1, CCR2b, CCR3, CCR8, CX3CR1/V28, gpr1,

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APJ, ChemR23 and RDC1, but the *in vivo* role of these coreceptors is unknown (reviewed by Clapham & McKnight, 2002).

One important role for virus coreceptor usage could be at transmission, in determining the first cell to become infected. We know that R5 HIV-1 replicates preferentially in the asymptomatic phase of infection. Moreover, primary intestinal epithelial cells seem to transfer R5 HIV-1 variants selectively to $CCR5^+$ cells (Meng *et al.*, 2002). In addition, vaginal infection of rhesus macaques with a mixture of chimeric simian/human immunodeficiency viruses having either an X4 or an R5 HIV-1 envelope results in selective transmission of viruses containing the R5 envelope gene (Lu *et al.*, 1996). Both intravaginal and intravenous infections of macaques with SIV conceivably lead to a major change in the intestinal lymphoid tissue. The $CD4^+$ T cell in the gut and other tissues throughout the body is the primary target for virus replication and acute infection is accompanied by an extensive loss of $CD4^+$ T cells within the first 2 weeks of infection (Hirsch *et al.*, 1998; Li *et al.*, 2005; Mattapallil *et al.*, 2005; Stahl-Hennig *et al.*, 1999; Veazey *et al.*, 1998; Zhang *et al.*, 1999). The initial steps of infection could conceivably be different depending on the route of transmission and this in turn could influence pathogenesis. Indeed, the influence of the route of transmission on pathogenic outcome has been widely discussed. Comparisons of disease progression in HIV-1 infection between injecting drug users and homosexual men have shown that the latter had a significantly accelerated progression rate (Eskild *et al.*, 1997; Pehrson *et al.*, 1997). On the other hand, Prins & Veugelers (1997) found little evidence for different disease progression between injecting drug users and homosexual men. Similarly, Hengge *et al.* (2003) found that the course of HIV disease does not depend on the mode of transmission. When looking at HIV-1 biological phenotype, however, Spijkerman *et al.* (1995) found a lower prevalence and incidence of the syncytium-inducing phenotype (now classified as CXCR4-using virus) among injecting drug users compared with homosexual men.

This prompted us to examine the possible influence of the route of infection on pathogenesis in a model system, using the same SIVsm inoculum virus (originating from a sooty mangabey), administered either intrarectally (IR) or intravenously (IV) to 30 cynomolgus macaques. The animals were monitored regularly for up to 5 years after infection, depending on the rate of disease progression. Sequential isolates were obtained on both hPBMCs and cynomolgus macaque PBMCs (mPBMCs) and tested for coreceptor use in the GHOST(3) and U87.CD4 cell lines, expressing CD4 and one coreceptor. We have shown here that particular coreceptor-usage patterns varied with the pathogenic outcome of SIV infection, but not according to the route of transmission. In addition, in a subgroup of monkeys, emergence of neutralization resistance was followed using both autologous and heterologous sera [see accompanying paper by Laurén *et al.* (2006) in this issue)].

METHODS

Animals and viruses. Thirty cynomolgus macaques were inoculated IV or IR with SIVsm (strain SMM-3 from P. Fultz and H. McClure, Yerkes National Primate Research Center, Atlanta, GA, USA). The animals were seronegative for SIV and simian T-lymphotropic virus and PCR-negative for simian retrovirus type D before entering the study. Housing and handling of animals followed the guidelines of the Swedish Ethical Committee for Animal Protection. Strain SMM-3 was originally isolated from a naturally infected sooty mangabey (Fultz *et al.*, 1986). The virus stock used for infection was propagated *in vitro* in cultures of mPBMCs (Quesada-Rolander *et al.*, 1996). Cell-free virus stocks (10 MID_{50}) were used for infection, which meant that animals infected by the IR route received an approximately 10^3-fold higher virus dose than the IV-inoculated animals. The monkeys were monitored for general clinical status. Blood samples for virus isolation and for viral load and $CD4^+$ T-cell count determinations were collected at regular intervals. Animals were kept until development of AIDS or, if asymptomatic, until the end of the study period, when they were euthanized (Table 1). In the present study, two to four isolates were tested from each monkey, depending on virus isolation frequency and the length of the monkey's survival time. The first isolate was usually obtained as early as 2 weeks post-infection (p.i.); the second isolate was obtained by 3 or 4 months; the third isolate was chosen at a time in between the second and the last isolate; and the last (fourth) isolate was collected at the time of euthanization (Table 2).

Virus isolation was performed by co-cultivation of mPBMCs with mPBMCs or with hPBMCs stimulated by phytohaemagglutinin (Nilsson *et al.*, 1995). Reisolates were passaged no more than twice. Cell-free supernatants were screened for the amount of reverse transcriptase (RT) with a Cavidi HS kit Lenti RT (Cavidi Tech) and stored at $-80\,^{\circ}C$ until use. SIV RNA levels in plasma were measured by using a highly sensitive quantitative competitive RT-PCR assay with a lower detection limit of 100 RNA equivalents $(\text{ml plasma})^{-1}$, as described in detail elsewhere (Ten Haaft *et al.*, 1998). Animals were monitored for changes in their $CD4^+$ cell counts by using two-colour flow-cytometric analysis as reported previously (Mäkitalo *et al.*, 2000). $CD4^+$ T-cell values were evaluated as the percentage of the total T-cell count. When observing the rate of change of $CD4^+$ lymphocytes, values obtained before infection were set as 100 % for each animal and following values were calculated in relation to these set-point values. The rate of change as a percentage of the $CD4^+$ lymphocyte population was fitted by linear-regression analysis.

HIV-2 strain 1010, known to use all of the coreceptors evaluated in this study (Mörner *et al.*, 1999), was used as a positive control in tests for coreceptor usage.

Determination of coreceptor usage. The GHOST(3) cell lines stably expressed the CD4 receptor and one of the chemokine receptors CCR3, CCR5, CXCR4, CXCR6 or the orphan receptor gpr15 (Mörner *et al.*, 1999). As a control, the parental cell line had been engineered to express the CD4 receptor alone and none of the coreceptors. Notably, the clone 3 cell line used in the experiments expressed a low but detectable level of endogenous CXCR4. GHOST(3) cells are stably transfected with the green fluorescent protein (GFP) gene driven by the $HIV\text{-}2_{ROD}$ long terminal repeat. Upon infection, the viral Tat protein activates GFP expression. The GHOST(3) cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7·5 % FBS and antibiotics (penicillin and streptomycin) in 25 cm^2 cell-culture flasks.

U87.CD4 cell lines expressing no coreceptor (parental) or expressing CCR3, CXCR4 or CCR5 (Deng *et al.*, 1997) were used in one experiment. U87.CD4 cells were maintained in DMEM supplemented with

Table 1. Summary of disease progression in macaques

Monkey	Group	Route of inoculation	End of study		CD4 decline (% CD4 per month)†			Virus isolation frequency (%)§
			Disease*	Time (months)	Whole period	0–3 months‡	4 months–end‡	
D24	P	IR	sAIDS	12	−8·1	−16·5	−2·5	88
C87	P	IR	sAIDS	12	−7·4	−15·6	−3·7	90
D23	P	IR	sAIDS	12	−7·0	−14·9	−5·4	88
B174	P	IV	sAIDS	15	−5·3	−14·6	−2·8	100
C83	P	IR	sAIDS	15	−4·5	−7·4	−5·1	91
D27	P	IR	sAIDS	18	−4·5	−30·1	−1·7	89
C45	P	IR	sAIDS	24	−3·9	−20·4	−2·1	93
C2	P	IR	sAIDS	15	−3·6	−20·0	−1·2	82
C73	P	IR	sAIDS	18	−3·3	−6·7	−3·1	83
C38	P	IR	Lymphoma, sAIDS	24	−3·3	−4·7	−4·1	92
C37	P	IR	sAIDS	15	−3·3	−14·6	−2·2	91
C86	P	IR	sAIDS	24	−3·1	−9·2	−1·6	93
D26	P	IR	sAIDS	18	−2·9	−17·1	−1·2	89
C27	P	IV	Weight loss, diarrhoea	18	−2·8	−4·7	−2·3	100
C26	P	IV	sAIDS	21	−2·7	−22·8	−0·3	100
C39	P	IR	Lymphoma, sAIDS	18	−2·6	−6·1	−3·9	58
56-3	SP	IV	None	38	−1·9	0·4	−2·7	75
C20	P	IV	sAIDS	18	−1·8	−8·6	−1·5	100
C24	SP	IV	None	38	−1·0	−18·1	−0·5	100
C68	SP	IR	None	53	−1·0	−10·9	−1·0	91
C54	SP	IR	Lymphoma, sAIDS	47	−0·9	−2·6	−1·4	71
59-3	SP	IV	None	36	−0·9	−13·6	0·0	62
B173	LTNP	IV	None	39	−0·8	−2·5	−0·8	31
C44	P	IR	sAIDS	27	−0·7	−10·9	−0·5	93
C82	LTNP	IR	None	35	−0·5	−3·3	−0·6	72
C93	LTNP	IR	None	50	−0·4	−4·3	−0·6	52
D25	LTNP	IR	None	39	−0·3	−12·2	−0·1	21
C1	LTNP	IR	Lymphopenia, weight loss	60	−0·2	−8·4	−0·3	15
C35	LTNP	IR	None	34	−0·04	−10·5	0·3	10
D28	LTNP	IR	None	39	0·5	−6·8	0·8	50
Median P ($n=18$)				18	−3·3			91
Median SP ($n=5$)				43	−1·0			75
Median LTNP ($n=7$)				NA	−0·3			31

*Symptoms of disease at end of study; sAIDS, simian AIDS.

†$CD4^+$ cell decline is presented as the regression coefficient of linear-regression analysis, taking into account 10–27 determinations per monkey.

‡The $CD4^+$ cell decline for two different time intervals p.i. was fitted with two separate regressions.

§Virus isolation frequency was the proportion of successful virus isolations, monitored approximately every 3 months throughout the lifespan of animals.

NA, Not applicable.

10 % FBS and antibiotics (penicillin and streptomycin) in 25 cm^2 cell-culture flasks. Cultures were incubated in a humidified atmosphere with 5 % CO_2 at 37 °C.

Infection of GHOST(3) and U87.CD4 cells was carried out as described previously (Björndal *et al.*, 1997; Vödrös *et al.*, 2001b). Briefly, 1 day before infection, cells were seeded in 24- or 48-well plates. Before infection, medium was replaced with 200 µl fresh medium containing 2 µg polybrene ml^{-1} and 300 or 150 µl undiluted virus was added to two identical wells of 24- or 48-well plates, respectively. Cultures were incubated overnight, washed with PBS and further incubated with fresh medium. Three days after infection, the GHOST(3)

Table 2. Coreceptor use of sequential SIVsm isolates isolated on hPBMCs

Monkey	Months after infection	Virus dose* [log_{10} (pg RT ml^{-1})]	Coreceptor usage†	
			Main coreceptor	Additional coreceptor
SIVsm strain SMM-3		3·2	CCR5	CXCR6, gpr15
Progressors				
D23	0·5	3·6	CCR5, CXCR6	gpr15
	3	3·7	CCR5, CXCR6, gpr15	
	12	3·8	CCR5, gpr15	CXCR6
C83	0·5	3·3	CCR5	gpr15
	3	2·1	CCR5	
	15	3·0	CCR5	
B174	0·5	3·9	CCR5, CXCR6	CCR3, CXCR4‡, gpr15, unknown§
	3	3·8	CXCR6, gpr15	CCR5
	12	3·6	CCR5	gpr15
	15	3·9	CCR5, gpr15	CXCR6
C27	0·5	3·7	CCR5	CXCR6, gpr15
	3	3·1	CCR5	CXCR6, gpr15
	12	3·5	CCR5	CXCR6, gpr15
	18	3·6	CCR5	gpr15
C2	0·5	ND	CCR5	CXCR6
	3	ND	CCR5	CXCR6
	9	ND	CCR5	CXCR6
	17	ND	CCR5	
C37	0·5	3·0	CCR5	
	3	2·7	CCR5	gpr15, CXCR6
	6	2·6	CCR5	
	15	1·8	Weak‖	
D27	0·5	3·1	CCR5	CXCR6
	4	3·0	CCR5	
	12	3·1	CCR5	CXCR6, gpr15
	18	2·3	CCR5	
C39	0·5	2·9	CCR5	gpr15
	3	2·3	CCR5	
	12	1·6	CCR5	
	18	2·9	CCR5	gpr15
C73	0·5	4·0	CXCR6	CCR5, CCR3, CXCR4, gpr15, unknown
	3	3·9	CXCR6	CCR5, CCR3, gpr15
	18	3·9	CCR5, CXCR6	CCR3, CXCR4, gpr15
C26	0·5	3·8	CCR5	CXCR6, gpr15
	3	3·7	CCR5	CXCR6, gpr15
	12	3·7	CCR5	CXCR6, gpr15
	18	3·7	CCR5	gpr15
C45	0·5	3·1	CCR5	gpr15
	3	3·0	CCR5	gpr15
	24	3·0	CCR5	
D26	0·5	3·6	CCR5	CCR3, CXCR4, CXCR6
	4	3·6	CCR5	CXCR4, CXCR6
	12	3·6	CCR5	CXCR6, gpr15
	18	3·6	CCR5	CXCR6, gpr15
C20	0·5	3·8	CCR5	CXCR6, gpr15
	3	3·8	CCR5	CXCR6, gpr15
	12	3·5	CCR5	CXCR6, gpr15
	18	3·7	CCR5	gpr15
C44	0·5	3·8	CXCR4	CCR5, CXCR6, gpr15
	3	3·9	CCR5, CXCR6, gpr15	CCR3, CXCR4, unknown

Table 2. cont.

Monkey	Months after infection	Virus dose* [log_{10} (pg RT ml^{-1})]	Coreceptor usage†	
			Main coreceptor	Additional coreceptor
	12	3·8	CCR5, gpr15	
	27	3·9	CCR5, gpr15	CXCR6
Progressors with evolution to X4 virus				
C38	0·5	3·0	CCR5	
	3	2·9	CCR5	
	12	2·8	CCR5	
	21	2·4	CCR5	CXCR4
C86	0·5	3·9	CCR5	CXCR6, gpr15
	3	3·9	CCR5	CXCR6, gpr15
	12	3·9	CCR5	CXCR6, gpr15
	24	3·2	CCR5	CXCR4, CXCR6, gpr15
C87	0·5	1·7	CCR5	
	3	3·3	CCR5	
	12	3·2	CCR5, CXCR6	CXCR4
D24	0·5	3·6	CCR5, CXCR6	gpr15
	3	3·5	CCR5	CXCR6, gpr15
	12	3·6	CXCR4	CXCR6
Slow progressors				
56-3	2	ND	CCR5	CXCR6, gpr15
	3	ND	CCR5	CXCR6, gpr15
	24	ND	CCR5	CXCR6
	38	ND	CCR5	CXCR6
C24	0·5	3·7	CCR5	CXCR6, gpr15
	3	3·7	CCR5, gpr15	CXCR6
	12	3·7	CCR5, gpr15	CXCR6
	36	3·4	CCR5, gpr15	CXCR6
59-3	2	ND	CCR5, CXCR6	CCR3, CXCR4
	3	ND	CCR5, CXCR6	CCR3, CXCR4
	24	ND	CCR5	CXCR6, gpr15
	37	ND	CCR5	
C68	0·5	4·0	CXCR6	CCR5, CCR3, CXCR4, gpr15, unknown
	3	4·0	CCR3, CXCR6	CCR5, CXCR4, gpr15, unknown
	30	3·7	CXCR6	CCR5, CCR3, CXCR4, gpr15, unknown
	53	4·0	CXCR6	CCR5, CCR3, CXCR4, gpr15, unknown
C54	0·5	2·3	CCR5	
	3	2·0	Weak	
	30	2·4	CCR5	
	46	2·9	CCR5	
Long-term non-progressors				
B173	0·5	3·9	CXCR6	CCR5, CCR3, CXCR4, gpr15, unknown
	3	3·9	CCR5, CXCR6	gpr15
	39	3·4	Weak	
C82	0·5	3·1	CCR5	
	3	1·6	Weak	
	15	2·4	CCR5	
	35	2·8	CCR5	
C93	0·5	3·0	CCR5	CXCR6
	4	2·4	CCR5	
	33	2·2	CCR5	
D25	0·5	3·9	CCR5	CXCR4, CXCR6, gpr15
	2	1·9	Weak	
C1	0·5	ND	CCR5, CXCR6, gpr15	

Table 2. cont.

Monkey	Months after infection	Virus dose* [$\log_{10}$ (pg RT ml^{-1})]	Coreceptor usage†	
			Main coreceptor	Additional coreceptor
	60	ND	CCR5	
C35	0·5	ND	CCR5, CXCR6	CCR3, CXCR4, gpr15
	3	ND	CCR5, CXCR6	CCR3
D28	0·5	2·9	CCR5	CXCR6
	4	3·1	CCR5	
	39	3·2	CCR5	CXCR6, gpr15

*Virus dose was measured as the amount of RT. ND, Not determined.

†Coreceptor usage was determined by flow-cytometric analysis of infected GHOST(3) cells. The results from the flow-cytometric analysis gave the RTCN value (see Methods), a quantitative measurement of coreceptor use by the tested virus (Vödrös *et al.*, 2001b). RTCN values of the main coreceptors were at least twice as high as with the additional coreceptors.

‡CXCR4 use was verified by inhibition with the CXCR4 antagonist AMD3100 (see Table 4).

§'Unknown' receptor refers to partial inhibition by AMD3100 (see Table 4).

||Indeterminate result.

cultures were observed with light and fluorescence microscopes and cells from one of the duplicate wells were treated with EDTA and harvested. The detached cells were fixed with paraformaldehyde at a final concentration of 2 %. Fixed cells were kept at 4 °C for at least 2 h before analysis by flow cytometry. Infected U87.CD4 cultures were screened for syncytia by using light microscopy at days 3 and 6 after infection. Culture supernatant from infected cultures was collected at day 6 from both U87.CD4 and GHOST(3) cells for viral-antigen detection. An in-house HIV-2/SIV capture ELISA was used to detect viral antigens produced by the infected cells (Thorstensson *et al.*, 1991).

The infected GHOST(3) cells were analysed with a flow cytometer (FACScan; Becton Dickinson). The results from the flow-cytometric analysis gave us a 'ratio to cell negative' (RTCN) value, a quantitative measure of the coreceptor usage by the tested virus (Vödrös *et al.*, 2001b). Briefly, $RTCN = (FI \times \%)_{virus\ inf.}/(FI \times \%)_{neg.\ control}$, where % is the proportion of fluorescence-positive cells and FI is the mean fluorescence intensity of the fluorescence-positive cells. Negative controls were uninfected cultures from the corresponding coreceptor-expressing cell line. An RTCN value of >10 was considered positive, indicating use of that particular coreceptor; an RTCN value of <5 was considered negative; values of between 5 and 10 remained indeterminate.

In a set of experiments, the CXCR4 antagonist AMD3100 was used to block CXCR4 on the GHOST(3) cells. AMD3100 was added to cells 5 min prior to infection in 200 µl culture medium at a concentration of 1 µg ml^{-1}.

Statistics. The different groups of monkeys were compared by using the Mann–Whitney non-parametric test, calculated by using SPSS statistical software.

RESULTS

Disease progression

Monkeys were divided into three groups based on the rate of disease progression, CD4 decline, time of death and, whenever available, viral load (Table 1, Fig. 1). The three groups were named progressor (P), slow progressor (SP) and long-term non-progressor (LTNP). There was no difference in either the CD4 decline or the viral load when comparing monkeys inoculated by different routes of infection. As expected, CD4 decline was more pronounced in the first 3 months of infection, whilst the decline of $CD4^+$ T cells was less thereafter. There was one exception to this general rule: monkey 56-3 did not seem to lose any CD4 cells for the first 3 months after infection, although CD4 counts declined thereafter. For the majority of animals, the pattern of viral load was consistent with the observations by Ten Haaft *et al.* (1998), in that a threshold plasma virus load that was greater than 10^5 RNA equivalents (ml plasma)$^{-1}$ at 6–12 weeks after inoculation could predict a faster disease progression. A threshold virus load value that remained below 10^4 RNA equivalents (ml plasma)$^{-1}$ was indicative of a non-pathogenic course of infection. Even though there was no difference in viral load according to route of infection, the isolates from IV-infected monkeys replicated to higher titres on hPBMCs than isolates from IR-infected monkeys ($P=0{\cdot}006$, Mann–Whitney test). This was also reflected by the fact that virus isolations were more frequently successful in the animals infected by the IV route.

Progressors. The P monkeys showed the fastest decline in $CD4^+$ T-cell count and all animals developed simian AIDS or AIDS-related symptoms (Table 1). Median CD4 decline in the P group was $-3{\cdot}34$ % $CD4^+$ T cells per month (range, $-8{\cdot}05$ to $-0{\cdot}69$ % $CD4^+$ T cells per month). Due to early disease symptoms, three of the monkeys (C87, D23 and D24) were euthanized 1 year after infection. Others developed symptoms of disease later, but all were euthanized by 27 months p.i. and the median time to AIDS in this group was 18 months. Virus isolation frequency was high, with values above 82 % (median, 91 %). Interestingly, all of the IV-infected monkeys had a virus isolation frequency of 100 %, even though their viral load was not exceptionally high. None of

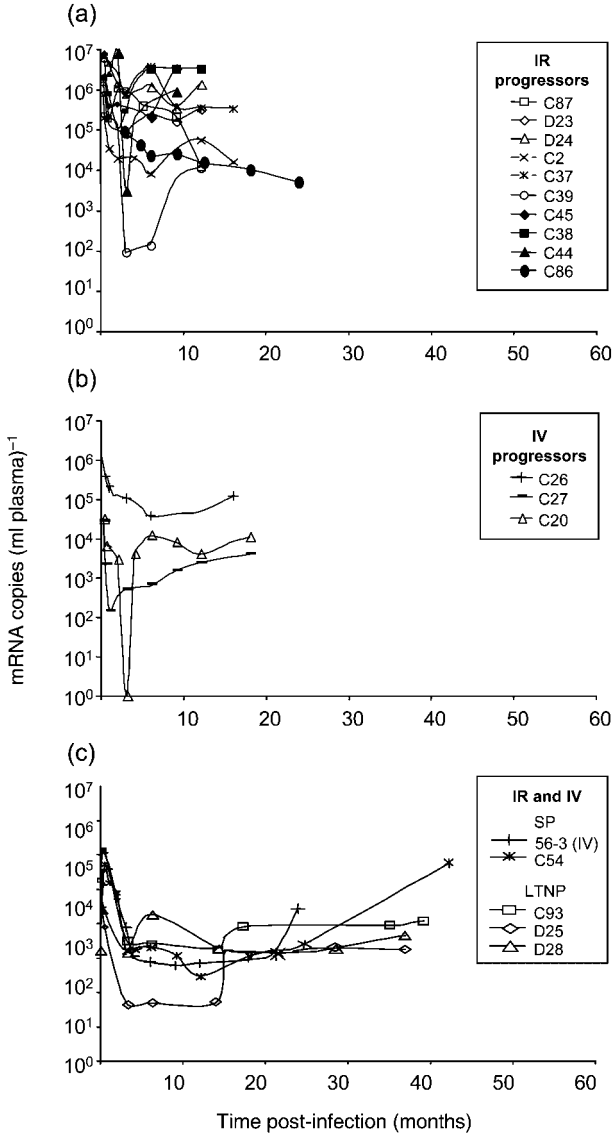

Fig. 1. Viral load in 13 (out of 18) P monkeys infected IR (a) or IV (b) and in two (out of five) SP and three (out of seven) LTNP monkeys (c).

the IR-infected monkeys had a virus isolation frequency of 100 % and one monkey (C39) had an unusually low virus isolation frequency (58 %). Monkey C39 was also exceptional for having a different pattern of plasma viral load (Fig. 1). In most of the P monkeys, plasma viral load was initially high [$>10^6$ RNA copies (ml plasma)$^{-1}$] and stayed high, whereas in monkey C39, viral load declined below 10^3 copies (ml plasma)$^{-1}$ within 3 months and then increased slowly again to values of $>10^4$ copies (ml plasma)$^{-1}$. Monkeys C20, C27 and C44 with the slowest CD4 decline in the P group also showed a drop in viral load pattern, but it increased again within 3 months; in the case of C20 and C44, viral load was above 10^4, whereas for monkey C27, the level was somewhat lower. Monkey C27 had to be euthanized at 18 months after infection because of extensive diarrhoea and weight loss and was therefore considered a P monkey.

Slow progressors (SP). Median CD4 cell decline in the SP group was −0·98 % $CD4^+$ T cells per month (range, −1·92 to −0·88 % cells per month) (Table 1). Four out of five monkeys in this group did not show disease symptoms during the study period (median 43 months), whilst one monkey developed lymphoma 47 months p.i. The other monkeys, although without signs of disease, showed a higher rate of CD4 cell decline (Table 1) and had a higher virus isolation frequency (median 75 %) than the monkeys in the LTNP group (median 31 %, Table 1). In the two SP monkeys (C54, 56-3) in which viral load was measured, initial viraemia declined sharply and remained low for many months (Fig. 1c). It should be remembered that monkey 56-3 was the only monkey with a stable CD4 count for the first 3 months after infection (Table 1). The SP group also fell between the P and LTNP groups with regard to the viral load parameter.

Long-term non-progressors. With one exception, the monkeys in the LTNP group did not show any symptoms of disease over the observation period of 34–60 months. Monkey C1 showed lymphopenia and weight loss by the time of autopsy at 60 months p.i. (Table 1). Whilst LTNP monkeys also showed a substantial CD4 decline for the initial 3–4 months after infection, the overall decline in the percentage of $CD4^+$ T cells was not particularly distinct (median CD4 decline of −0·26 % $CD4^+$ T cells per month). Overall, the relative number of $CD4^+$ cells in this group remained close to the CD4 values before infection for a long period of time. Late in infection, the CD4 values decreased to slightly below the CD4 values before infection. Virus isolation from LTNPs was unsuccessful at many time points and varied between 10 and 72 % for individual monkeys. The viral load of the LTNP group decreased after the early peak viraemia to values of between 10^3 and 10^4 RNA copies (ml plasma)$^{-1}$ and remained stable over the entire study period (Fig. 1c).

Coreceptor use of sequential SIVsm reisolates

Isolation on hPBMCs. Coreceptor usage of 105 isolates from 30 monkeys was tested on GHOST(3) cells (Table 2). All isolates, with one exception, infected cells via CCR5, and in general CCR5 was the most efficiently used coreceptor. CCR5 was defined as the main coreceptor used in most cases (70 out of 105 isolates), based on RTCN values at least twice as high as with other coreceptors. In another 20 cases, CCR5 was used as efficiently as CXCR6 or gpr15. The reisolates could infect CXCR6- or gpr15-expressing cells in 60 and 55 % of the cases, respectively, and 46 % of the isolates used all three receptors. The 12 month reisolate from monkey D24, which did not use CCR5 at all, used CXCR4 efficiently as the major coreceptor (RTCN ranged from 107 to 244 and was tested

in three independent experiments). In addition, ten isolates derived from three P monkeys (five isolates), one SP (four isolates) and one LTNP (one isolate) used CXCR6 (six isolates), CXCR6 in combination with CCR3 or gpr15 (two isolates) or used the CXCR4 (two isolates) receptor more efficiently than CCR5 (Table 2).

The effect of virus quantity on infection was analysed in two different ways. We performed dilution experiments with three different viruses and found that, within a 16-fold dilution range, the most efficiently used coreceptors could not be diluted out (Fig. 2). We also plotted the virus dose used for infection of GHOST(3) cell lines, expressed as concentration of RT (pg ml^{-1}) against the number of coreceptors used by a particular isolate (Fig. 3). Whilst the majority of isolates had RT activity corresponding to the 3 log_{10} pg RT ml^{-1} range (69 %), there were a few isolates containing <3 log_{10} pg RT (21 % had 2 log_{10} pg RT ml^{-1} and 7 % had 1 log_{10} pg RT ml^{-1}). Such comparison was important, as some of the animals did not yield more virus than 2 log_{10} pg RT ml^{-1} in the virus stocks from PBMCs. Interestingly, all three monkeys diagnosed with lymphoma (C38, C39 and C54) and two out of seven LTNP monkeys (C82, C93) yielded low-titre virus. Characteristically, such viruses used CCR5 as the major, and most often the only, coreceptor.

We observed some interesting patterns of change in coreceptor usage over time (Table 2). In LTNP monkeys, coreceptor usage narrowed or stable CCR5 usage (R5 virus) dominated in six out of seven cases. The same pattern was seen in three out of five SP animals. A minority of P monkeys showed the narrowing pattern (7/18 cases), whereas the remaining animals showed broadening of coreceptor use (3/18), stable coreceptor use of several coreceptors (1/18) or fluctuation between the different coreceptor-usage patterns (7/18).

We also compared monkeys infected by the mucosal or IV route of infection with regard to the number of coreceptors used. There was no difference in how many coreceptors were used according to route of infection (Mann–Whitney test).

Isolation on mPBMCs. Wherever cells were available from the infected monkeys, viruses were reisolated on mPBMCs. A total of 11 isolations was carried out. Eight of these were from samples exactly matching the time of isolations on hPBMCs, whilst the remaining three samples were 2 or 4 months apart (with some samples from monkeys C73 and D24). Similar to isolates obtained on hPBMCs, the major coreceptor used was CCR5 (Table 3). CXCR6, less frequently CCR3 and in one case gpr15 were used as additional coreceptors. The majority of isolates induced GFP in parental, CCR3- and CXCR4-expressing cells to a similar extent. Therefore, first we inhibited the CXCR4 background on the GHOST(3) cells with AMD3100. Interestingly, AMD3100 could not inhibit GFP induction in these cases, suggesting that the cells might have expressed an unidentified receptor that could serve as an entry factor for many of the mPBMC isolates. To rule out the possible effect of the unidentified receptor on the results, we tested coreceptor usage of isolates on

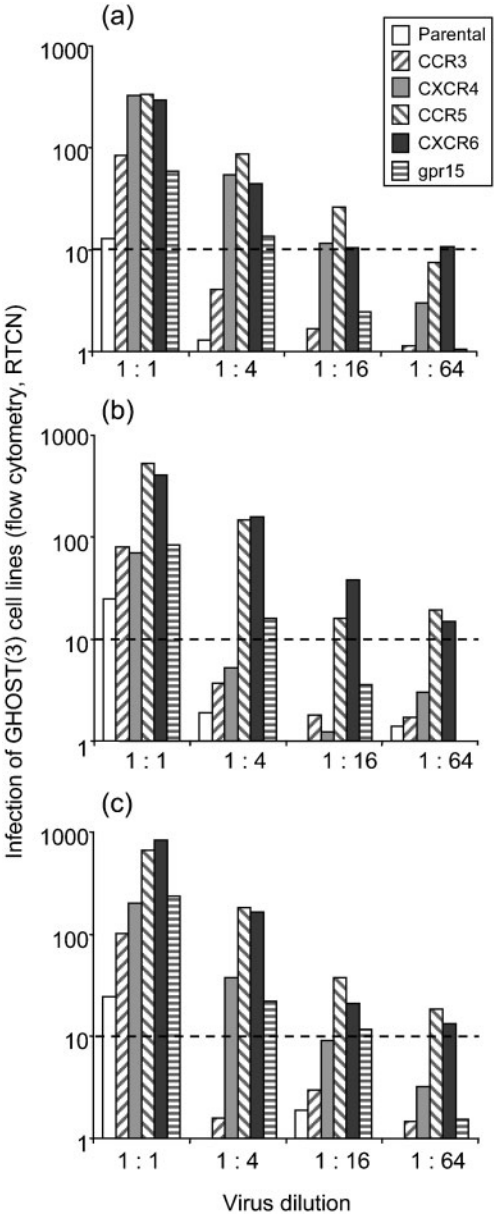

Fig. 2. Effect of virus dose on the detection of coreceptor use. SIVsm isolated on hPBMCs 2 weeks after infection from monkeys C73 (a), C68 (b) and B173 (c). GHOST(3) cell lines were infected with four different virus doses. The starting virus dose (1 : 1) for infection according to the concentration of RT was 3·8 log_{10} pg RT ml^{-1} (a), 4·2 log_{10} pg RT ml^{-1} (b) and 3·8 log_{10} pg RT ml^{-1} (c) and fourfold dilution steps were carried out. The results of coreceptor usage of isolates were determined by flow-cytometric analysis of infected GHOST(3) cells. An RTCN value of above 10 was considered positive, indicating use of that particular coreceptor. An RTCN value of below 5 was considered negative. Values between 5 and 10 were indeterminate. See Methods for details of RTCN value calculation.

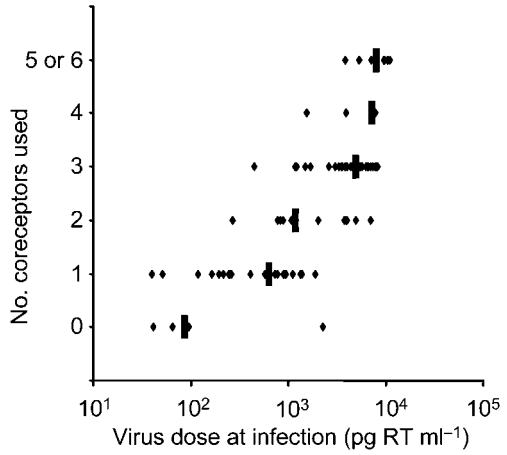

Fig. 3. Amount of RT (pg ml^{-1}) in virus stocks used for infection of GHOST(3) cells in relation to the pattern of coreceptor use. ◆, Individual isolates; |, median per group. For details of coreceptor usage, see Table 2.

another indicator cell line, U87.CD4, expressing CCR3, CCR5, CXCR4 or no coreceptor (parental cells). When a low level of syncytium induction and viral-antigen production in U87.CD4 parental cells was observed, it was taken as background and compared with cells engineered to express coreceptors. In this way, we confirmed CCR3 use, but excluded CXCR4 use by the mPBMC isolates.

A comparison of five exact matches of isolates derived on hPBMCs and mPBMCs and with virus doses for GHOST(3) and U87.CD4 cells of RT amounts in the 3 $\log_{10}$ pg RT ml^{-1} range revealed differences in coreceptor usage (Fig. 4). Both sets of isolates used CCR5 and CXCR6 regularly, whereas the use of CCR3 was more variable in both cases. Furthermore, only one mPBMC isolate included in the present comparison was able to use gpr15, whereas the hPBMC counterparts could use this coreceptor. In this comparison of exact matches of isolates, three of the hPBMC isolates could infect cells using CXCR4, whereas none of the viruses isolated on mPBMCs could use the CXCR4 coreceptor. Conversely, isolates derived from mPBMCs were able to use an additional, unidentified coreceptor on GHOST(3) cells more often than isolates from hPBMCs.

Detection of CXCR4 use among SIVsm reisolates from hPBMCs

In spite of the rare detection of CXCR4-using SIV isolates reported, we could recover CXCR4-using viruses from 13 monkeys when isolated on hPBMCs. Two patterns of CXCR4 use could be demonstrated. CXCR4 use either appeared early during the acute phase of infection and disappeared later, or CXCR4 use only appeared late in infection during immunodeficiency (Table 2). Whilst the first pattern has been encountered in an earlier work (Vödrös *et al.*, 2001a, 2003), the late appearance of CXCR4 use, reminiscent of HIV-1 infection, has not been demonstrated in SIV infection. In this study, CXCR4-using virus could be

Table 3. Coreceptor use of sequential SIVsm isolates isolated on mPBMCs

Monkey	Months after infection	Virus dose [$\log_{10}$ (pg RTml^{-1})]*	Coreceptor usage†	
			Main coreceptor	Additional coreceptor
Progressors				
C73	5	3·5	CCR5	CXCR6, CCR3, unknown‡
	7	3·5	CCR5	CXCR6, CCR3
	18	3·3	CCR5	CXCR6, CCR3, gpr15
D24	0·5	3·6	CCR5	CXCR6, CCR3, unknown
	3	3·4	CCR5	CXCR6, unknown
	10	3·3	CCR5	CXCR6, CCR3
Slow progressors				
C68	0·5	3·4	CCR5	CXCR6, CCR3, unknown
	30	2·7	CCR5	
	53	2·6	CCR5	
Long-term non-progressors				
B173	0·5	3·6	CCR5	CXCR6, unknown
	39	2·7	CCR5	CXCR6, unknown

*Virus dose was measured as the amount of RT.

†Coreceptor usage was determined by flow-cytometric analysis of infected GHOST(3) cells. In addition, tests on U87.CD4–CCR3, –CCR5, –CXCR4 and parental cell lines were also performed. The results from the flow-cytometric analysis of GHOST(3) cells gave the RTCN value (see Methods). RTCN values of the main coreceptors were at least twice as high as with the additional coreceptors.

‡'Unknown' receptor refers to no inhibition by AMD3100.

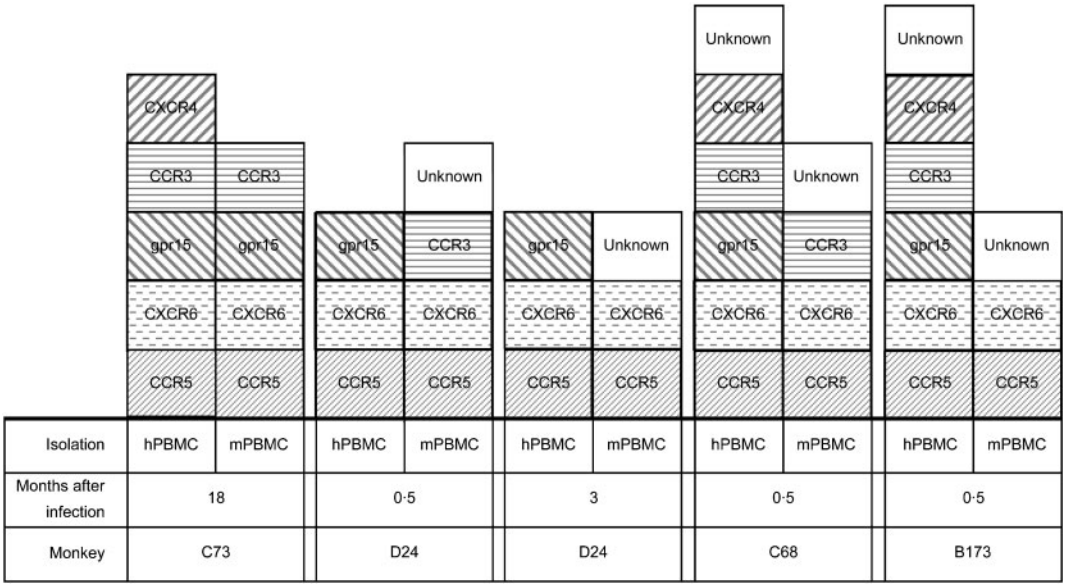

Fig. 4. Comparison of coreceptor usage of isolates derived from hPBMCs and mPBMCs. Isolates were exact matches according to time of isolation and with RT activity corresponding to 3 $\log_{10}$ pg RT ml^{-1}.

isolated on hPBMCs from four animals with progressive disease (C38, C86, C87 and D24). Interestingly, all four animals were infected by the IR route. Sequential isolates from one macaque (C73) showed fluctuation in CXCR4 use: CXCR4-using virus was detected 2 weeks after infection and disappeared later on, but was isolated again at the time of immunodeficiency. There were monkeys in all three groups from which virus with CXCR4 usage could be isolated shortly after infection. Remarkably, three out of seven animals in the LTNP group harboured CXCR4-using virus initially. In all three cases, CXCR4 usage disappeared and we observed a general narrowing of coreceptor usage over time.

With regard to the endogenously expressed CXCR4 on GHOST(3) cells, it was important to confirm that isolates indeed used CXCR4 and that CXCR4 use of multitropic isolates did not interfere with the use and detection of other receptors. Therefore, we carried out a set of experiments in which we blocked CXCR4 on the cells by adding a CXCR4 antagonist, AMD3100, to the cultures prior to infection. CXCR4 use of early reisolates was inhibited by AMD3100 (86–97 % inhibition) in four out of five macaques tested (Table 4). In one monkey, the CXCR4 usage of the early isolate was inhibited by AMD3100 only to 54 %. The emerging CXCR4 use by late reisolates could also be verified and, in addition, the inhibition of CXCR4 on the cells did not interfere with the alternative coreceptor (CXCR6 and gpr15)-usage pattern. In line with our previous observations (Vödrös *et al.*, 2001a, 2003), in some cases, we could not achieve complete inhibition of CXCR4 use by AMD3100 (B173, B174, C68; Table 4). This indicated that some of the viruses, similar to the viruses isolated on mPBMCs, may use an additional coreceptor also present on GHOST(3) cells or that the SIV envelope interaction with CXCR4 is different from the interaction between envelopes of HIV-1 and CXCR4, as suggested by Owen *et al.* (2000).

DISCUSSION

Here, we evaluated coreceptor usage of SIVsm isolates obtained from a large number of IV- or IR-infected SIVsm monkeys with different rates of disease progression. There was no difference in how many and which coreceptors were used according to route of infection. All isolates but one used CCR5 for cell entry. Moreover, CCR5 was the major coreceptor used in 70 out of 105 reisolates tested. A comparison of exact matches of isolates derived on hPBMCs and mPBMCs showed that CCR5 and CXCR6 usage was similar. However, we could isolate CXCR4-using viruses on hPBMCs, but not on mPBMCs, suggesting that hPBMCs may select for variants with CXCR4 usage.

CCR5 has been shown to be the major coreceptor for SIV and is essential for cell entry in combination with CD4 (Chen *et al.*, 1997; Edinger *et al.*, 1997; Marcon *et al.*, 1997). In addition, CXCR6 and gpr15 have been shown to be common coreceptors for SIV (Alkhatib *et al.*, 1997; Deng *et al.*, 1997; Farzan *et al.*, 1997). Our results are in agreement with previous studies on sequential SIV isolates where CCR5 has been shown to be an important coreceptor for both early and late SIVsm and SIVmne variants (Kimata *et al.*, 1999a; Vödrös *et al.*, 2001a). However, impaired ability to use gpr15 by SIVmac had no effect on infection of rhesus macaques, indicating that gpr15 plays a minor role in pathogenicity *in vivo* (Pöhlmann *et al.*, 1999). Likewise, we

Table 4. Inhibition of CXCR4 receptors on GHOST(3) cells using AMD3100 with isolates from hPBMC isolation

Monkey	Progression	Months after infection	AMD3100	Receptors (RTCN value)*					
				CD4	CD4+ CCR3	CD4+ CXCR4	CD4+ CCR5	CD4+ CXCR6	CD4+ gpr15
Early reisolates									
B174	P	0·5	−	52	31	371	1644	841	330
			+	49	19	32	1736	828	325
C73	P	0·5	−	20	8	205	740	311	145
			+	16	6	6	579	367	183
C44	P	0·5	−	15	35	57	26	40	8
			+	9	19	3	39	23	1
C68	SP	0·5	−	22	14	32	844	998	149
			+	27	11	15	1001	854	194
B173	LTNP	0·5	−	74	50	421	3315	1968	662
			+	90	49	61	2959	2533	740
Late reisolates									
C87	P	12	−	7	37	214	195	819	36
			+	8	39	1	266	969	15
D24	P	12	−	70	164	107	34	47	6
			+	3	4	6	3	27	6

*Coreceptor usage of viruses isolated on hPBMCs was determined by flow-cytometric analysis of infected GHOST(3) cells. An RTCN value of above 10 was considered positive, indicating use of that particular coreceptor. An RTCN value of below 5 was considered negative. Values between 5 and 10 were indeterminate. See Methods for details of RTCN value calculation.

found only one gpr15-using virus out of 11 when isolated on mPMBCs. CXCR4 use for SIV has been a rare phenomenon and in at least two studies was demonstrated with virus isolated and/or passaged on hPBMCs (Owen *et al.*, 2000; Schols & de Clercq, 1998; Vödrös *et al.*, 2001a).

The fast selection of CXCR4-using virus within one virus isolation and a maximum of two passages on hPBMCs is highly interesting. SIV adaptation to human cells has been described previously and was associated with a shorter form of gp41 (gp31) (Hirsch *et al.*, 1989). In our case, SIV might use a coreceptor that is advantageous during growth on hPBMCs and, because of this, may acquire CXCR4 use during isolation on hPBMCs. This may not be surprising, as we know that CXCR4 is expressed at higher levels than CCR5 in hPBMC cultures (Bleul *et al.*, 1997). However, there is no evidence that the *in vivo* distribution of CCR3, CCR5 and CXCR4 differs between humans and macaques (Zhang *et al.*, 1998). Comparison of 11 rhesus macaque chemokine receptors with their human counterparts showed close similarities of 98·9 % (CCR5) and 99·4 % (CXCR4) (Margulies *et al.*, 2001). The similarity of CXCR6 was somewhat lower, 96·8 %, but, in our hands, SIVsm envelopes fused with human CCR5, (CXCR6) and gpr15 as efficiently as with rhesus counterparts (Vödrös *et al.*, 2003).

A considerable number of our isolates, from both hPBMCs (eight isolates) and mPBMCs (six isolates), could also infect the GHOST(3) parental cell line, even in the presence of the CXCR4 antagonist AMD3100. This is in line with our previous observations that complete inhibition of CXCR4 usage by SIVsm could not be achieved by AMD3100, indicating that these viruses may use an additional coreceptor also present on GHOST(3) cells (Vödrös *et al.*, 2001a). Forte *et al.* (2003) have also suggested the possibility that SIVmne variants that are able to infect PBMCs lacking CCR5 use an unidentified coreceptor. Another possibility could be that the SIV envelope interaction with CXCR4 is different from the interaction of the HIV-1 envelope and CXCR4, as suggested by Owen *et al.* (2000).

It has been shown previously that different HIV-1 and SIV isolates can interact with receptors in different ways (Antonsson *et al.*, 2003; Edinger *et al.*, 1997; Karlsson *et al.*, 2003). Macrophage-tropic SIV has been shown to depend on both the N-terminal and the second extracellular loop (ECL-2) of CCR5 for cell entry, whilst T cell-tropic SIV isolates require only CCR5 ECL-2 (Edinger *et al.*, 1997). A general comparison of SIVsm and HIV-1 may be difficult. However, it has been suggested that ECL-2 is important for CXCR4-using HIV-1, whilst viruses of the R5 phenotype vary in their requirement for binding to the N terminus of CCR5 (Brelot *et al.*, 1997; Doranz *et al.*, 1999; Karlsson *et al.*, 2004). By using receptor chimeras between CCR5 and CXCR4, Karlsson *et al.* (2004) showed that the R5 phenotype of HIV-1 undergoes evolution over time and changes the mode of CCR5 usage. Also, it has been shown that CXCR4-using HIV-1 seems to undergo evolution *in vivo* and shows decreasing sensitivity to CXCR4 antagonists (Stalmeijer *et al.*, 2004), suggesting that a change in the binding capacity to ECL-2 occurs. In support of this, it is

known that the CXCR4 antagonist AMD3100 binds to ECL-2 (Gerlach *et al.*, 2001; Hatse *et al.*, 2001; Labrosse *et al.*, 1998). Conceivably, SIV may also change in the infected macaques over time and, as a result, late virus isolates may differ from early isolates and inoculum virus in the mode of receptor usage. Such a change may lead to altered tropism for different cell types. Indeed, late SIV isolates are often more cytopathic than early isolates, further supporting the possibility that SIV also changes throughout infection (Kimata *et al.*, 1999a, b; Rudensey *et al.*, 1995).

Although we could not isolate CXCR4-using viruses on mPBMCs, a significant number of SIVsm viruses isolated on hPBMCs used CXCR4 efficiently in this study. On the one hand, we were able to isolate CXCR4-using virus on hPBMCs from seven macaques 2 weeks after infection, but not beyond 3 months, confirming our previous findings (Vödrös *et al.*, 2001a). On the other hand, the finding that virus with CXCR4 usage could be isolated from four macaques late in disease, at the time of immunodeficiency, was new and unexpected. In one P animal (monkey C73), CXCR4-using virus was isolated both early and late in infection, but not in between. The last isolate of monkey D24 had a unique phenotype, X4X6, using CXCR4 as the major coreceptor and not using CCR5 at all (Table 2). Viruses from the remaining three monkeys showed evolution to CXCR4 use, although use of CCR5 and CXCR6 was more efficient than CXCR4 use. When large populations of HIV-1 subtype B-infected individuals are considered, the appearance of CXCR4 usage has been estimated to involve 50 % of AIDS cases (Björndal *et al.*, 1997; Karlsson *et al.*, 1994). Large variations between groups and also according to subtype have been reported. For example, in HIV-1 subtype C infections, the X4 phenotype occurred less frequently (17 % of the AIDS cases; Cilliers *et al.*, 2003). In our SIVsm material, overall 5/18 monkeys (28 %) in the P group yielded CXCR4-using viruses.

A further similarity between HIV-1 and SIVsm can be found by considering the overall pattern of coreceptor usage in relation to the pathogenic process. The majority of P monkeys yielded virus with a broadening of coreceptor usage, stable use of several coreceptors or fluctuation between different coreceptor-usage patterns over time. In LTNP monkeys, coreceptor usage of isolates narrowed from multitropic to CCR5 use only, or, when CCR5 was the only coreceptor used, this remained stable over the entire observation period. We have previously evaluated the coreceptor usage of sequential isolates from six SIVsm-infected cynomolgus macaques with progressive disease and found a narrowing pattern of coreceptor usage, in that late isolates obtained at the time of immunodeficiency often could not establish a productive infection in gpr15- or CXCR6-expressing cells (Vödrös *et al.*, 2001a, 2003). In the present study, we found a similar pattern in seven P monkeys. However, if considering the large number of animals (18 P monkeys) used in the present study, the majority of monkeys with progressive disease could use multiple coreceptors effectively. Taken together, our results indicate that SIV may evolve in the infected host, resulting in changes in the mode of coreceptor usage to include the use of CXCR4 or a similar receptor.

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