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Coreceptor Usage of Sequential Isolates from Cynomolgus Monkeys Experimentally Infected with Simian Immunodeficiency Virus (SIVsm)

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Sequential isolates from eight cynomolgus monkeys experimentally infected with simian immunodeficiency virus (SIVsm, of sooty mangabey origin) were tested for coreceptor use in the human osteosarcoma indicator cell line, GHOST(3), expressing CD4 and one or another of the chemokine receptors CCR3, CCR5, CXCR4, BOB, or the orphan receptor Bonzo. The indicator cell line carries the human immunodeficiency virus type 2 long terminal repeat-driven green fluorescence protein gene that becomes activated upon infection with HIV or SIV and fluorescence can be quantitated by flow cytometric analysis. The methodological details are described in the accompanying paper (Vödrös *et al.,* 2001, *Virology* 290, in press). All SIVsm inoculum viruses and reisolates used CCR5 with a high level of efficiency. CCR5 use was stable over time. BOB and Bonzo use was less efficient than CCR5 use and, in particular, late isolates obtained at the time of immunodeficiency varied greatly in their coreceptor use and often could not establish a productive infection in BOB- or Bonzo-expressing cells. Unexpectedly, early reisolates obtained 12 days postinfection could infect the entire GHOST(3) panel including the parental cells. In one case this was due to use of CXCR4, either transfected or endogenously expressed on the GHOST(3) cells. Our results demonstrate the complex coreceptor use of SIVsm isolates. Moreover, they focus attention on the initial phase of virus replication when the availability of target cells may govern the replication pattern of the virus.

Key Words: early SIV replication; immunodeficiency in macaques; coreceptor usage; GHOST(3) indicator cells; BOB; Bonzo.

INTRODUCTION

Simian immunodeficiency virus (SIV) infection in monkeys has many similarities to human immunodeficiency virus (HIV) infection in humans. Using the SIV model in macaques we may explore the critical steps in disease progression following infection with a lentivirus causing immunodeficiency. Earlier studies have shown that the main receptors for HIV-1 entry into target cells are the chemokine receptors CCR5 and CXCR4 in conjunction with CD4 (Deng *et al.,* 1996; Dragic *et al.,* 1996; Feng *et al.,* 1996). HIV-1 isolates previously called slow/low or non-syncytium-inducing (NSI) use CCR5, whereas viruses able to infect established T cell lines, and formerly called rapid/high or syncytium-inducing (SI), use CXCR4 as coreceptor for cell entry (Björndal *et al.,* 1997; Scarlatti *et al.,* 1997). CXCR4 using HIV-1 isolates are often characterized by dual tropism or multi-tropism and may use CCR5, CCR3, and CCR2b as well. Like HIV-1, SIV isolates regularly use CCR5 as coreceptor for cell entry (Chen *et al.,* 1997; Edinger *et al.,* 1997; Marcon *et al.,* 1997) but, unlike HIV-1, use of CXCR4 is rarely found and is largely dependent on the virus strain used (Owen *et al.,* 2000; Schols and De Clercq, 1998).

Studies on SIVMne infection in macaques showed that viral phenotypic changes may occur over time (Rudensey *et al.,* 1995, 1998). These changes involved neutralizing antibody recognition, syncytium formation, and macrophage tropism of viruses isolated from macaque peripheral blood mononuclear cells (PBMC) at different time points. Virus isolates obtained at the time of AIDS were resistant to neutralization, induced syncytia in the CEMx174 cell line, and were poorly infectious for macaque macrophages. These phenotypic changes suggested that SIV, like HIV-1, evolves over the course of pathogenic infection (Scarlatti *et al.,* 1997).

Our earlier studies on eight experimentally SIVsm- (of sooty mangabey origin) infected macaques showed that reisolates obtained various times after infection were frequently resistant to neutralization with autologous sera (Zhang *et al.,* 1993). These antigenic changes suggested that phenotypic changes, such as changes in coreceptor use, may occur in the virus populations over time. In the present study we therefore tested coreceptor use of sequential SIVsm isolates from the same eight cynomolgus macaques differing in the rate of disease progression. In addition to CCR5, SIV has been shown to

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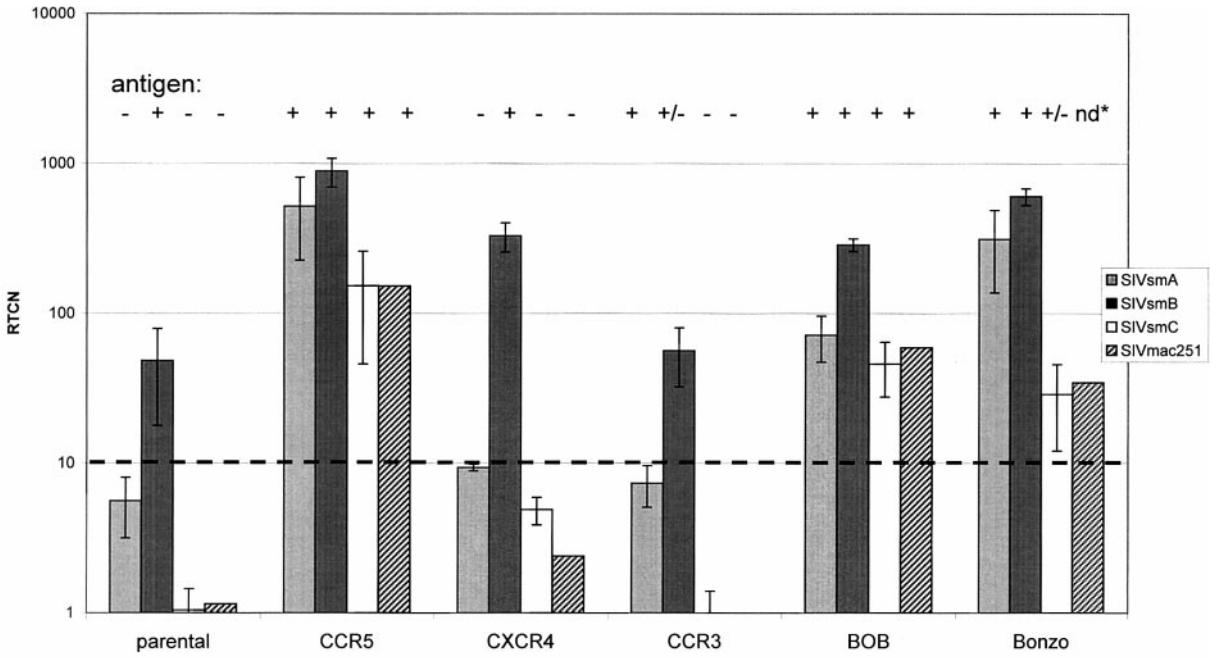

FIG. 1. Coreceptor use of inoculum viruses. GHOST(3) cell lines were infected with 56–96 ID_{50}. The columns indicate the fold difference from uninfected control cells (for definition of RTCN see Materials and Methods) and represent the mean of two independent experiments. The experimental variation is shown by the bars at the top of each column. SIVmac251 was tested only once. Results of viral antigen production: +, both tests positive; −, both tests negative; ±, one test positive, the other negative; *, not determined.

use the orphan receptor BOB/gpr15 and Bonzo/STRL33/TYMSTRL (Deng *et al.,* 1997; Edinger *et al.,* 1998a; Farzan *et al.,* 1997), recently identified as a transmembrane CXC chemokine receptor (Matloubian *et al.,* 2000). BOB and Bonzo use is rare among HIV-1 isolates. A series of cell lines with stable expression of CD4 and one or another of the orphan receptor BOB or the chemokine receptors CCR3, CCR5, CXCR4, or Bonzo was available from the GHOST(3) series (Cecilia *et al.,* 1998; Mörner *et al.,* 1999) and was used in the present experiments. In the accompanying paper (Vödrös *et al.,* 2001) we describe the validation of the GHOST(3) indicator system for use with HIV-1. In the present work we used the same indicator cell system and asked the question of whether changes in coreceptor use of SIVsm reisolates occur over time.

RESULTS

Coreceptor usage of SIVsm inoculum viruses

Entry of viruses into the indicator cells expressing different coreceptors was measured by the extent of green fluorescence protein (GFP) induction. The ability to establish productive infection following entry was estimated by viral antigen production in the culture fluids 6 days postinfection.

Regardless of *in vitro* passage history, all inoculum viruses used CCR5, BOB, and Bonzo, like uncloned SIVmac (Fig. 1). (For a detailed history of inoculum viruses see Materials and Methods.) The experimental variation of the extent of GFP induction, characterized by the calculated RTCN value (ratio to cell negative; see details of calculation under Materials and Methods) and indicated by the bars on the top of each column, did not exceed 0.75 log. In fact, RTCN values for a given virus–receptor combination repeatedly scored positive (or were repeatedly negative). Remarkably, SIVsmB, but not SIVsmA and C, was able to infect CXCR4-expressing cells. It must be remembered that SIVsmB had been maintained in passage in human peripheral blood mononuclear cells (hPBMC) for 3 months prior to use in the present experiments. Since cultured hPBMC express CXCR4 more abundantly than CCR5 (Bleul *et al.,* 1997), the SIVsmB virus could have been selected for CXCR4 use. In line with this, SIVsmB scored positive on the parental GHOST(3) cells as well, known to have a low level of spontaneous expression of CXCR4. This may also account for the apparent CCR3 use by this virus. Interestingly, SIVsmC with the shortest passage history in hPBMC was the least able to use CXCR4.

Viruses that scored positive for coreceptor usage by GFP induction could also establish a productive infection in the corresponding GHOST(3) cell lines, as shown by antigen production in supernatant culture fluids (Fig. 1). It must be noted that SIVsmA in CCR3-expressing cells showed indeterminate RTCN but produced antigen, albeit small amounts, in both experiments. On the other hand, SIVsmB with positive RTCN on CCR3-expressing

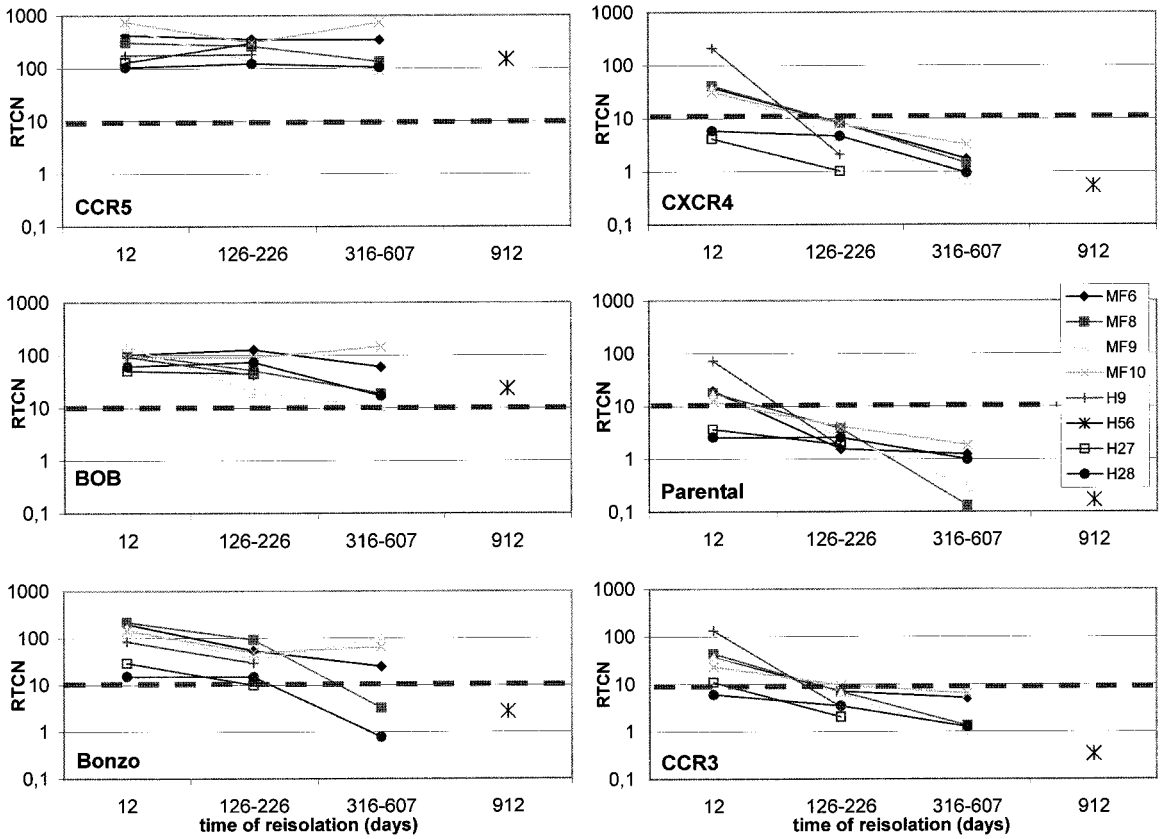

FIG. 2. Coreceptor use of 20 reisolates obtained from experimentally infected macaques over time.

cells and SIVsmC on Bonzo-expressing cells produced viral antigen in one of two experiments only.

Coreceptor usage of sequential reisolates

We tested 3 isolates each from five monkeys, 2 isolates each from two monkeys that died within 6 months postinfection, and the 1 isolate that could be recovered from a long-term survivor monkey: altogether 20 isolates. Four monkeys (MF6, MF8, MF9, and MF10) were infected with the SIVsmA virus, whereas SIVsmB and SIVsmC were inoculated to two monkeys each (H9, H56 and H27, H28, respectively).

Entry, Measured as GFP Induction. The first reisolates obtained 12 days postinfection used CCR5, BOB, and Bonzo as coreceptors, like the inoculum viruses (Fig. 2). As expected, virus reisolated from the SIVsmB-infected monkey (H9) appeared to use CXCR4 and CCR3 as well, like the SIVsmB inoculum virus. However, it was unexpected that 12-day reioslates from monkeys inoculated with SIVsmA—which itself gave indeterminate RTCN values on CXCR4- and CCR3-expressing cells—induced GFP in all indicator cells including the GHOST(3) parental cells (Fig. 2). Even the first reisolate from monkey H27, inoculated with SIVsmC, gave positive results on CCR3-expressing cells, although the RTCN was 10.9, just above the cut-off level (Fig. 2).

The capacity to induce GFP in CXCR4-expressing cells decreased over time and was in all cases absent when reisolates obtained shortly before the animals' death from immunodeficiency were tested (Fig. 2). Similar results were obtained on the parental cells and with two exceptions (indeterminate results of the third isolates from monkeys MF6 and MF10) on the CCR3-expressing cell line. In contrast, CCR5 use and, to some extent, BOB use were stable during infection and reisolates obtained at different times after infection seemed to use these coreceptors with similar high efficiency (Fig. 2). The capacity to use Bonzo showed minor variations (1.15 log) among the 12-day reisolates but diverged later on (2.15 log variation before the death of animals). In fact, the late reisolates from monkeys MF8 and H28 lost their ability to use Bonzo. The only isolate available from monkey H56, obtained 912 days postinfection, used CCR5 and BOB as coreceptors for cell entry.

Productive Infection, Measured as Antigen Production in Culture Supernatants. Infection of CCR5-expressing cells resulted in production of large amounts of antigen in the majority of cases (Table 1). Antigen production from the parental GHOST(3) cells or cells expressing CXCR4, CCR3, BOB, or Bonzo varied according to the time of reisolation (data not shown). Accordingly, cultures infected with the 12-day reisolates produced virus in

TABLE 1

Viral Antigen Production in Fluorescence-Positive Cultures[a]

	CCR5		BOB		Bonzo	
Antigen OD_{490}[b]	% of isolates	Mean RTCN (SD)	% of isolates	Mean RTCN (SD)	% of isolates	Mean RTCN (SD)
>3	45	391.9 (226.4)	0	—	0	—
1.5–2.99	23	140.5 (97.2)	0	—	0	—
0.5–1.49	23	203.3 (116.9)	14	59.7 (14.6)	26	320.3 (64.4)
0.1–0.49	9	111.4 (29.1)	68	77.7 (43.8)	42	119.7 (49.9)
<0.1	0	—	18	28.9 (20.9)	32	65.7 (39.8)

[a] Data from 22 isolates for CCR5- and BOB-expressing cells and from 19 virus isolates for Bonzo-expressing cells that gave RTCN values >10.
[b] Supernatant culture fluids were assayed for viral antigen by ELISA. The figures denote optical density (OD) measured at 490 nm.

most cases. In contrast, late reisolates, even if able to induce GFP, did not produce antigen in any but CCR5-expressing cells. This indicates that early reisolates showed higher replicative capacity in the GHOST(3) indicator cells than late reisolates. The results also show that GFP induction in GHOST(3) cells is more sensitive for detection of SIVsm infection than antigen production.

Inhibition of CXCR4-using viruses with AMD3100

To estimate the contribution of endogenous CXCR4 expression on virus entry, we treated GHOST(3) cells with the specific CXCR4 antagonist, AMD3100, prior to infection. CXCR4 use of a control virus, a HIV-1 isolate of R5X4 phenotype, was effectively inhibited by AMD3100 (Fig. 3). In this case the RTCN value decreased from 146 to 1.3 in exogenous CXCR4-expressing cells and from 33 to 1.8 in parental cells, resulting in a 2.04 and 1.28 log inhibition, respectively. AMD3100 also inhibited CXCR4 use by the first reisolate from monkey H9 (H9:12) and the corresponding SIVsmB inoculum virus. The reduction in RTCN values was 1.08 and 1.12 log, respectively, in CXCR4-expressing cells, decreasing RTCN to a still pos-

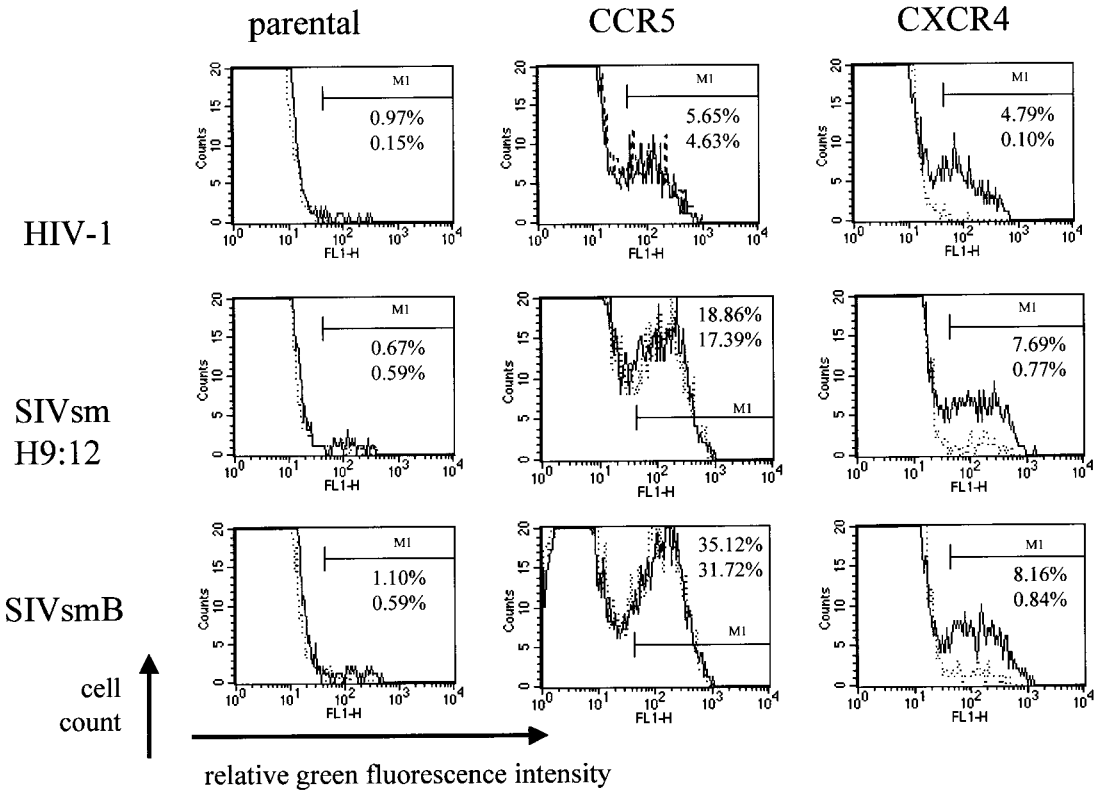

FIG. 3. Inhibition of virus infection by AMD3100 (1 μg/ml). GFP induction in GHOST(3) parental, CCR5- or CXCR4-expressing cells analyzed by flow cytometry 3 days postinfection. The control HIV-1 virus is a dual-tropic R5X4 isolate. The proportion of fluorescence-positive cells without (top number) and with AMD3100 (bottom number) is indicated in each diagram. Continuous line: infection in the absence of AMD3100, discontinuous line: infection in the presence of AMD3100. M1 denotes 0.1% fluorescence-positive cells in the uninfected control cultures.

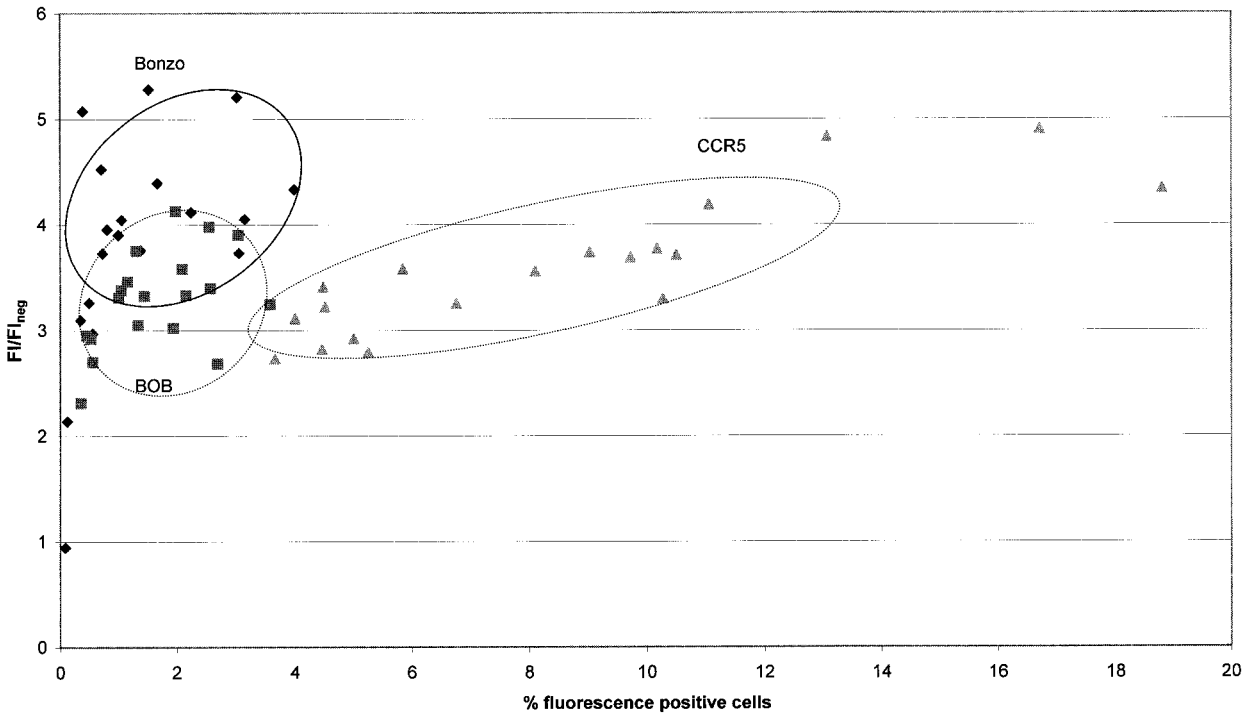

FIG. 4. Comparison of the efficiency of CCR5 (▲), Bonzo (◆), and BOB (■) use as a function of the percentage of fluorescence positive cells (x) and their fluorescence intensity (y).

itive value (19.8 and 19.4, respectively). However, the drug proved ineffective on infection of the parental cells with these viruses (Fig. 4). Compared to the complete inhibition of CXCR4 use of HIV-1, infection by SIVsmB and H9:12 was only partially inhibited by AMD3100. This result suggests that a CXCR4-using virus can be recovered early after infection from a monkey infected with a virus that was able to use CXCR4. In addition, SIVsmB and the H9:12 isolates seem to be able to use another—as yet unidentified—coreceptor, as suggested by the partial inhibition obtained with AMD3100. Whether CXCR4 or another unidentified receptor was expressed on the GHOST(3) cells, the capacity to use any of these receptors was reduced over time in the macaques.

Infection via CCR5 is more effective than via the BOB or Bonzo coreceptors

As shown in Figs. 1 and 2, all three inoculum viruses and the 20 reisolates from eight monkeys used CCR5 and BOB and, with the exception of 3, also used Bonzo as coreceptor for cell entry. Interestingly, the proportion of fluorescence-positive cells was higher on GHOST(3).CCR5 cells than on BOB- or Bonzo-expressing cells (Fig. 4). GHOST(3).CCR5 cultures also produced larger amounts of viral antigen (Table 1) in that about 90% of the viruses produced viral antigen with optical density (OD) values higher than 0.5. In contrast, infection with the same viruses resulted in a high level of viral antigen production in only 14% of BOB- and 26% of Bonzo-expressing cell cultures. This indicates that many of the fluorescent GHOST(3).BOB and Bonzo cultures did not produce virus or produced very little virus (OD $<$ 0.5). Discordant GFP induction and antigen production were particularly striking with reisolates obtained at the time of overt immunodeficiency. In fact, only 12-day reisolates produced amounts $>$0.5 OD from Bonzo-expressing cells, similarly to the inoculum viruses. The results indicate that SIVsm inoculum viruses and reisolates efficiently use CCR5 and establish a productive infection following entry into cells. In contrast, infection by the BOB or Bonzo coreceptors is less efficient, and the capacity to establish a productive infection decreases over time.

DISCUSSION

Our results show that viruses reisolated from cynomolgus macaques following intravenous inoculation of SIVsm use CCR5, BOB, and Bonzo as coreceptors for cell entry. In addition, and depending on the passage history of the inoculum virus, CXCR4 use could be demonstrated with one inoculum virus and a corresponding early reisolate. Interestingly, CXCR4 use was only partially inhibited by AMD3100, a specific CXCR4 antagonist, when these two isolates were tested, suggesting the use of another receptor that remains to be identified.

Even if early reisolates induced GFP in parental or CXCR4- and CCR3-expressing GHOST(3) cells, subsequent reisolates gradually lost this ability, suggesting that this phenotype is not favored or may even be selected against. One possibility is that the initial target

cells for SIVsm replication had decreased in number or had been eliminated as a consequence of virus replication. Veazey *et al.* (1998) found that activated memory T-lymphocytes in the lamina propria of the intestinal epithelium are the initial target cells for SIVmac replication. Infection of rhesus monkeys resulted in a profound and selective depletion of $CD4^+$ T-cells in the intestine during the first 2–3 weeks of infection (Smit-McBride *et al.,* 1998; Veazey *et al.,* 1998). It is conceivable that depletion of the activated memory $CD4^+$ cells causes a shortage in the preferred target cells available for virus replication. If so, this would explain the initial decrease in viral load, particularly in tissues (Veazey *et al.,* 1998). Following initial depletion of the preferred target cells, other cells, like naive lymphocytes and macrophages, available at the same site or other sites, may express receptors for inflammatory chemokines, in the first place CCR5. It has been shown that the cytokine environment regulates cytokine receptor expression and occupancy and, as a consequence, also determines the susceptibility of macrophages to HIV or SIV infection (Wang *et al.,* 1998; Wu *et al.,* 1997). Indeed, early inflammatory responses involving RANTES production in tissues of SIV-infected macaques have been described (Cheret *et al.,* 1999). In addition, phenotypic changes in peripheral blood monocytes of infected macaques have also been described (Otani *et al.,* 1998; Zhou *et al.,* 1999), perhaps leading to an increase in the availability of CCR5-expressing cells. According to this scenario, lymphocytes that are continuously recruited to the organized lymphoid tissue become activated by antigenic stimulation and provide new target cells for SIV infection. However, these new target cells express CCR5 and favor replication of CCR5-using viruses. This would then predict profound changes in the biological phenotype of SIV replicating initially and at later times. Moreover, the early phase of SIV infection seems to be crucial in establishing the *in vivo* replication pattern and associated clinical course in infected macaques. Lifson *et al.* (1997) suggested that host factors that exert their effects prior to development of specific immune responses, like susceptibility of macaque cells to infection (measured *in vitro* in their experiments), are critical in determining the set points for subsequent plasma viremia and thereby determine progression rate. In this respect, it is noteworthy that HIV-1-infected individuals have a significantly greater percentage of CCR5- than CXCR4-expressing $CD4^+$ T-cells in blood as compared to normal controls (Ostrowski *et al.,* 1998).

Recent findings show that the Tat protein, secreted by HIV-1-infected cells, is a specific CXCR4 antagonist that selectively inhibits the entry and replication of X4, but not R5, viruses in PBMC and may influence the early *in vivo* course of disease (Xiao *et al.,* 2000). It is possible that Tat is an antagonist also for the as yet undefined coreceptor used by our isolates. If so, this would explain the decreasing efficiency of the use of this latter coreceptor.

An alternative scenario takes into consideration the immune response of the host. Mattapallil *et al.* (1998) detected larger numbers of SIV-positive cell during primary acute and terminal stages of SIV infection compared to the asymptomatic stage. It is conceivable that in the very early, acute phase of infection, at the time of high-level viral replication, even CXCR4-using viruses are allowed to replicate in the naive $CD4^+$ cells, known to express CXCR4 (Bleul *et al.,* 1997). In a few weeks an effective immune response (including T-cell activation) is generated against the virus (Mattapallil *et al.,* 2000), causing a decrease in the number of infected cells. Conceivably, the immune response in conjunction with the phenotype of the available target cells may exert a selective pressure on the virus population. Consequently, the coreceptor usage pattern of the initially replicating viruses may differ from that of the viruses encountered later on.

No consistent change in coreceptor use preceding or concurring with immunodeficiency could be observed in our experiments. This is in line with SIVMne infection where no change in the ability of reisolated virus to use CCR5 (Rudensey *et al.,* 1998) or CCR5 and BOB (Kimata *et al.,* 1999) could be observed. Indeed, the high level of efficiency of CCR5 use by the SIVsm reisolates was remarkably constant over time, regardless of the disease progression rate of our macaques. Similarly to Edinger *et al.* (1998a), we also found that CCR5 use is more efficient than either BOB or Bonzo use. In our hands the capacity to use BOB was maintained but it became less efficient over time, since the ability of the reisolates to establish productive infection in the GHOST(3) cells was highest early in infection and decreased later. Bonzo use changed to an even larger extent than BOB use, in that reisolates obtained from three animals 1–2 years postinfection lacked the capacity to use Bonzo altogether. Characteristically, GFP induction-positive/antigen production-negative cultures were obtained upon infection with late reisolates. Chackerian *et al.* (1997) have suggested that interactions among the coreceptor(s), the viral envelope, and another, not yet identified, viral gene product may govern post-entry steps of viral replication. In view of the extensive genetic variation of SIV in infected macaques (Campbell and Hirsch, 1994; Hirsch *et al.,* 1990, 1991; Johnson and Hirsch, 1992; Kodama *et al.,* 1993; Mori *et al.,* 1992), this hypothetical viral gene is also likely to vary. If so, this could explain the increase in the proportion of discordant cultures with late reisolates.

It is once again interesting to note how *in vitro* passage history influences coreceptor use of SIVsm isolates (Hirsch *et al.,* 1989). Clearly, prolonged passage in human PBMC selected for virus able to use CXCR4. We know today that CXCR4 expression is more abundant in hPBMC cultures than CCR5 expression (Bleul *et al.,*

1997) and this may favor replication via the CXCR4 receptor. SIVsmB is a clear example of a CXCR4-using virus. This is in agreement with the findings of Hoffman *et al.* (1998), who infected macaques with simian HIV (SHIV) containing a X4 HIV-1 Env protein. The results show that CXCR4 can function as a coreceptor in macaques even though it is rarely used by simian immunodeficiency virus. Based on our results we may suggest that the CXCR4-using phenotype is not favored or may even be selected against *in vivo*. The best example is monkey H9, which was infected with SIVsmB and yielded a CXCR4-using virus early after infection but not 6 months later.

Taken together, our results show that changes in coreceptor use of SIVsm occur during pathogenic infection. Isolates from the initial phase of virus replication from five of seven monkeys tested were able to infect all GHOST(3) sublines; however, this ability was gradually lost over time. Since this pattern was similar in all monkeys tested, regardless of serum neutralizing activity to autologous virus, we suggest that the availability of target cells governs the replication pattern of the virus. CXCR4-using virus could be isolated early after infection from a monkey inoculated with an isolate able to use CXCR4. CCR5 use of viruses recovered from the animals was highly efficient and stable over time, perhaps reflecting the profound and long-standing inflammatory response concomitant with SIVsm infection. Our continued efforts are focused on the identification of the suspected unknown receptor. Candidate receptors may be Apj, Gpr1, ChemR23, and RDC1 (Choe *et al.,* 1998; Edinger *et al.,* 1998a,b; Eva and Sprengel, 1993; Methner *et al.,* 1997; Mörner *et al.,* 1999; Owman *et al.,* 1997; Shimizu *et al.,* 2000; Singh *et al.,* 1999; Zhang and Moore, 1999) and will be probed for their relevance in SIVsm pathogenesis.

Although obtained in the macaque model, our results may be relevant for HIV-1 pathogenesis and, once again, focus attention on the phase of intial virus replication, which might be critical in determining disease progression. The monkey model has the advantage of allowing dissection of this early phase of infection.

MATERIALS AND METHODS

Animals and viruses

The SIVsm (strain SMM-3) isolate was kindly provided by P. Fultz and H. McClure, Yerkes Regional Primate Research Center (Atlanta, GA). It was originally isolated from a naturally infected sooty mangabey monkey (*Cercocebus atys*) (Fultz *et al.,* 1986). This virus had been grown on the human T-cell line, HUT-78, followed by two passages in hPBMC (Putkonen *et al.,* 1989). Cell-free supernatant of hPBMC cultures, harvested 8 days after infection, was the source of inoculum A. Inoculum B was derived from inoculum A by further passage on hPBMC for 3 months. Inoculum C was derived from a cynomolgus macaque that had been infected with inoculum A and developed diarrhea, weight loss, and tachypnea 245 days postinoculation. Virus was isolated from the monkey's PBMC by cocultivation with hPBMC and the cell-free supernatant of this coculture was used as inoculum C. SIVmac251-infected HUT-78 cells were obtained from M. Essex (Harvard Medical School, Boston, MA). The HIV-1 control virus used in the inhibition experiments was a Cameroonian subtype B/D isolate, known to use both CCR5 and CXCR4 coreceptors (Tscherning-Casper *et al.,* 2000).

Eight cynomolgus monkeys were inoculated intravenously, four with SIVsmA (MF6, MF8, MF9, MF10), two with SIVsmC (H27, H28), and two with SIVsmB (H9, H56) (Zhang *et al.,* 1993). Two monkeys (H9 and H27) were fast progressors and died of immunodeficiency within 6 months, whereas two monkeys (H28 and H56) were long-term survivors and were sacrificed with immunodeficiency after 39 and 30 months, respectively. Seven monkeys (MF6, MF8, MF9, MF10, H27, H28, and H9) had lymphadenopathy and weight loss (4–35%). Monkeys MF6 and MF10 had neurological symptoms; monkey MF9 had diarrhea. Monkey H56 did not show any clinical symptoms up to 730 days postinfection but developed immunodeficiency afterward. After a transient decrease, the CD4 cell count returned to normal levels in this monkey. In contrast, in the fast progressor monkeys (H9 and H27) the CD4 level decreased rapidly and permanently.

Blood samples were collected from the monkeys at regular intervals and virus isolations were carried out 12, 126–226, and, if possible, 316–607 days after infection. In the case of monkey H56, the only virus isolate available was obtained 912 days postinfection. In total 20 isolates from eight monkeys were available and tested along with the three inoculum viruses for coreceptor use. Reisolates were passaged on hPBMC no more than three times. Cell-free supernatants from the hPBMC (Weber *et al.,* 1996) cultures were titrated on hPBMC, and 252 ID_{50} ($\pm$158) (infectious dose-50) were used for infection of GHOST(3) cells.

Cell lines

Human osteosarcoma cell lines, GHOST(3) engineered to stably express the CD4 receptor, and one or another of the chemokine receptors CCR3, CCR5, CXCR4, Bonzo, or the orphan receptor BOB were used (Cecilia *et al.,* 1998; Mörner *et al.,* 1999). The parental cell line had been engineered to express CD4 but none of the coreceptors. However, clone 3 used in our experiments is known to have a low level of spontaneous expression of CXCR4. The cells were stably transfected with the gene of the GFP driven by the HIV-2_{ROD} long terminal repeat (LTR). In case of infection, when the virus enters the cells by using CD4 and the given coreceptor, the viral

Tat protein becomes expressed and transactivates transcription of the GFP gene by the LTR. GFP expression in infected cells is easily detected in a fluorescence microscope and by flow cytometry. The GHOST(3) cell lines were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Palsley, Scotland) containing 7.5% fetal calf serum (Life Technologies, Karlsruhe, Germany) and antibiotics and incubated in a humidified atmosphere with 5% CO_2 at 37°C. Cells were detached by treatment with 5 mM EDTA solution and the cultures were split 1:15 twice a week.

Infection of GHOST(3) cells

One day before infection 24-well plates were prepared with 2–3 $\times$ 10^4 cells/well in 1 ml medium. Before infection, medium was replaced with 200 μl fresh medium and virus was added to duplicate wells in a volume of 300 μl/well. Two hours after infection, medium was added up to 1 ml/well. After an overnight incubation, cells were washed with PBS (PBS Dulbecco's, Life Technologies, Palsley, Scotland), 1 ml medium was added to each well, and the plates were further incubated. Three days after infection cultures were examined in a fluorescence microscope and cells from one of the parallel wells were harvested and prepared for flow cytometry by adding paraformaldehyde to a final concentration of 2% for at least 2 h. Remaining wells were split 1:5–1:10 and cultures were maintained for another 3 days for viral antigen detection by ELISA.

In one series of experiments the specific CXCR4 antagonist AMD3100 was used (Schols *et al.,* 1997). AMD3100 was added to the cells prior to infection in 200 μl medium at a concentration of 1 μg/ml and virus was added 5 min later. Parallel wells without inhibitor were included in each experiment and treated in the same way.

Flow cytometry

Flow cytometric analysis and its validation are described in the accompanying paper (Vödrös *et al.,* 2001). Briefly, infected GHOST(3) cells were detached from the wells by EDTA treatment, fixed by paraformaldehyde, and then analyzed by flow cytometry (FACScan, Beckton Dickinson). The GHOST(3) cell population was selected on the side scatter–forward scatter diagram, and the fluorescence intensity of 1.5 $\times$ 10^4 GHOST(3) cells was measured. For quantitative determination of the efficiency of coreceptor use a characteristic number, RTCN (ratio to cell negative), was calculated for each virus–coreceptor combination

$$\mathrm{RTCN} = (\mathrm{FI} \times \%)_{\mathrm{virus}}/(\mathrm{FI} \times \%)_{\mathrm{neg.control}},$$

where % is the proportion of fluorescence-positive cells and FI is the mean fluorescence intensity of the fluorescence-positive cells. Negative controls were mock-infected cultures from the corresponding coreceptor-expressing cell line. RTCN values above 10 scored positive, indicating use of the particular coreceptor. RTCN below 5 scored negative, while a RTCN value between 5 and 10 was considered indeterminate. The results obtained by flow cytometry strictly correlated to those of microscopy. For details of evaluation see the accompanying paper (Vödrös *et al.,* 2001).

SIV antigen detection

To detect viral antigens produced by the infected cells an in-house HIV-2/SIV capture enzyme-linked immunosorbent assay (ELISA) was used (Thorstensson *et al.,* 1991). In brief, cell-free supernatants from the cultures were added to 96-well microtiter plates previously coated with purified immunoglobulin-G from an asymptomatic HIV-2-positive blood donor and blocked with 1% bovine serum albumin. Captured SIV antigen was detected with rabbit anti-SIVmac serum followed by a horseradish peroxidase-conjugated swine and anti-rabbit immunoglobulin. Subsequently, the *o*-phenylenediamine substrate with H_2O_2 was added. The reaction was stopped with 2.5 M H_2SO_4 and the optical density was measured at 490 nm wavelength (OD_{490}). Viral antigen production was scored positive when OD_{490} values were above an absorbance value of 0.1.

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