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CD4-Independent Infection by HIV-2 Is Mediated by Fusin/CXCR4

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Summary

Several members of the chemokine receptor family have been shown to function in association with CD4 to permit HIV-1 entry and infection. However, the mechanism by which these molecules serve as CD4-associated cofactors is unclear. In the present report, we show that one member of this family, termed Fusin/CXCR4, is able to function as an alternative receptor for some isolates of HIV-2 in the absence of CD4. This conclusion is supported by the finding that (1) CD4-independent infection by these viruses is inhibited by an anti-Fusin monoclonal antibody, (2) Fusin expression renders human and nonhuman CD4-negative cell lines sensitive to HIV-2-induced syncytium induction and/or infection, and (3) Fusin is selectively down-regulated from the cell surface following HIV-2 infection. The finding that one chemokine receptor can function as a primary viral receptor strongly suggests that the HIV envelope glycoprotein contains a binding site for these proteins and that differences in the affinity and/or the availability of this site can extend the host range of these viruses to include a number of CD4-negative cell types.

Introduction

Although all isolates of HIV-1, HIV-2, and SIV have been shown to bind to CD4 during infection, other cellular factors are required for viral entry (Maddon et al., 1986; Hoxie et al., 1988; Clapham et al., 1992; Dragic et al., 1995). Recently, the CC chemokine receptor CCR5 was shown to function with CD4 as an entry cofactor for macrophage tropic (M-tropic) strains of HIV-1 (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996), while the CXC chemokine receptor Fusin (recently termed CXCR4) was shown to serve as a cofactor for virus isolates that are tropic for T cell lines (T-tropic) (Berson et al., 1996; Feng et al., 1996). Expression of CCR5 or Fusin in conjunction with human CD4 renders cells susceptible to infection by M- and T-tropic viruses, respectively. M-tropic virus strains appear to be responsible for sexual transmission and are prevalent during the asymptomatic period of infection (Zhu et al., 1993). The emergence of T-tropic strains of HIV-1 in some individuals correlates with a rapid decline in CD4 cell number and progression to AIDS (Schuitemaker et al., 1992; Connor and Ho, 1994). In addition to CCR5 and Fusin, other molecules in the chemokine receptor family, including CCR3 and CCR2b, function as cofactors for some HIV-1 isolates (Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996). Furthermore, some virus strains, such as the dual-tropic isolate 89.6, can use multiple cofactors and may represent an evolutionary intermediate between M- and T-tropic viruses (Doranz et al., 1996).

Chemokine receptors are seven transmembrane (7tm) domain, G protein–coupled molecules that mediate the chemotaxis of T cells and phagocytic cells to areas of inflammation (Power and Wells, 1996). While CCR5 expression appears to be largely restricted to cells of the hematopoietic lineage (Alkhatib et al., 1996; Deng et al., 1996; Samson et al., 1996), Fusin is expressed on a wide variety of CD4-positive and -negative cells in a number of tissues including brain, lung, and spleen (Federsppiel et al., 1993; Loetscher et al., 1994). Thus, during the course of HIV-1 infection, changes in the utilization of particular cofactors may enable viruses to infect an increasing number of CD4-positive target cells. However, while the utilization of these molecules following CD4 binding is likely to have an important role in determining the tropism of particular viral isolates, the mechanism by which chemokine receptors interact with CD4 and the viral envelope glycoprotein during infection is unclear. While it has been hypothesized that the envelope glycoprotein may associate with one or more of these 7tm proteins following CD4 binding (Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Feng et al., 1996), a direct interaction between these molecules has not been shown.

In addition to studies of CD4-dependent infection, several reports have shown that some HIV isolates can infect lymphoid (Clapham et al., 1992; McKnight et al., 1994) and nonlymphoid cells (Tateno et al., 1989; Ikeuchi et al., 1990; Harouse et al., 1991; Clapham et al., 1992)

in the absence of CD4. These studies are likely to be relevant to increasing evidence in humans (Wiley et al., 1986; Moses et al., 1993; Bagasra et al., 1996; Livingstone et al., 1996) and nonhuman primates (Mankowski et al., 1994; Dean et al., 1996) that CD4-negative cells can be targets for HIV infection in vivo. In general HIV infection of CD4-negative cells proceeds slowly and without cytopathic effects, most likely indicating the relatively inefficient use of an alternative receptor. Such is the case for the glycolipid galactosylceramide, which can serve as an alternative receptor on human glial and colon carcinoma cell lines (Harouse et al., 1991). However, particularly for some isolates of HIV-2, infection of CD4-negative cells can occur rapidly and with extensive cell fusion (Clapham et al., 1992). The highly cytopathic nature of these infections has suggested that these isolates are able to utilize one or more alternative receptors with high efficiency. However, the identity of these receptors has remained unknown.

In the present study, we show that isolates of HIV-2 that are able to infect a number of CD4-negative cells utilize the HIV-1 cofactor, Fusin, as a primary receptor in the absence of CD4. We show that the highly cytopathic, CD4-independent infection exhibited by these viruses is inhibited by a novel monoclonal antibody to Fusin and that the introduction of Fusin alone into a variety of human and nonhuman CD4-negative cells renders them fully permissive for HIV-2-induced syncytium induction and infection. We also show that the cloned HIV-2 *env* gene induced syncytium formation of nonhuman cells that expressed Fusin in the absence of CD4. Finally, as described for numerous viral receptors, we demonstrate that Fusin is selectively down-regulated from the cell surface following infection by these HIV-2 isolates. These findings strongly suggest that the HIV envelope glycoprotein contains a binding site for chemokine receptor proteins and that differences in the affinity and/or the availability of this site can markedly extend the host range of these viruses to include a number of CD4-negative cell types.

Results

Derivation of an HIV-2 Variant That Infects Cells in the Absence of CD4

To study receptors involved in CD4-independent infection, a biological variant of HIV-2, termed HIV-2/vcp, was derived from the HIV-2/NIH-z isolate (Zagury et al., 1988) (see Figure 1). HIV-2/vcp was shown to infect a number of CD4-negative lymphoid cell lines of T- (BC7, HSB, CEMss4−) and B- (Daudi and Nalm6) cell origin (Figure 1A) as well as the nonlymphoid rhabdomyosarcoma line RD (not shown). As expected, no infection was seen when these cell types were inoculated with the T cell line–tropic isolate HIV-1/LAI (Figure 1A). In addition, anti-CD4 monoclonal antibodies (MAbs) were not able to inhibit infection of BC7 (Figure 1B), Daudi or Nalm6 cells (not shown), or cell-to-cell fusion between HIV-2/vcp-infected cells and uninfected BC7 cells (Figure 1C). Similar to other HIV-2 isolates that can infect CD4-negative cells (Clapham et al., 1992), HIV-2/vcp infected the majority of these cell types with extensive cell fusion and killing, indicating the highly efficient use of one or more alternative receptors (not shown).

12G5, a Monoclonal Antibody That Inhibits CD4-Independent Infection by HIV-2

In an effort to analyze interactions between cellular molecules and viral envelope glycoproteins, an anti-cellular MAb termed 12G5 was derived that was able to inhibit syncytium induction by HIV-2/vcp on CD4-negative cell lines (see Experimental Procedures and Figure 2A). This MAb also inhibited syncytium induction by CP-MAC, a biological variant of SIVmac that has been shown to be highly infectious and fusogenic for Sup-T1 cells in a CD4-dependent manner (LaBranche et al., 1994) (Figure 2A). 12G5 was shown by FACS analysis to react with several CD4-positive and -negative human hematopoietic cell lines (Table 1) and with unfractionated human peripheral blood lymphocytes (not shown). Reactivity was also seen with some nonhematopoietic human cell lines including HeLa (cervical carcinoma) and RD (rhabdomyosarcoma), but not HOS (osteosarcoma) or U87 (glial) cells (Table 1). No reactivity was seen on the murine myeloma line SP2 or the other nonhuman cell lines shown in Table 1.

In addition to inhibiting cell fusion, 12G5 also neutralized infection by HIV-2/vcp and CP-MAC when preincubated with appropriate target cells. CP-MAC infection of Sup-T1 cells was readily inhibited by antibody concentrations of 1 μg/ml while somewhat higher concentrations (5–20 μg/ml) were required to inhibit HIV-2/vcp infection of CD4-negative lines including BC7, Nalm6, and Daudi (Figure 2B). Infection of these cell types was not inhibited by IgG_{2a} monoclonal antibodies that were isotype matched for 12G5 (not shown). Although 12G5 frequently caused cell clumping (Figure 2A), no inhibitory effects were seen on cell growth and no toxic effects were apparent when cells were cultured in antibody concentrations as high as 50 μg/ml (not shown).

12G5 also inhibited two other HIV-2 isolates, HIV-2/Rod-A and HIV-2/Rod-B, that have been shown to induce fusion on CD4-negative cell lines including Daudi and RD (Clapham et al., 1992). HIV-2/Rod-A infectivity has been shown to be enhanced by preincubation with soluble CD4 (sCD4) while HIV-2/Rod-B induces syncytia on these CD4-negative cells without sCD4. As shown in Table 2, 12G5 inhibited cell fusion at concentrations $\geq$5 μg/ml when H9 cells chronically infected by HIV-2/Rod-A or /Rod-B were cultured in the presence or absence of sCD4, respectively. Inhibition was also seen of another HIV-2 isolate, HIV-2/CBL-23 that induces syncytia on RD cells following preincubation with sCD4 (Table 2) (Clapham et al., 1992). Therefore, in addition to its anti-viral effects on CP-MAC and HIV-2/vcp, 12G5 inhibited infection and/or cell fusion for two other genetically distinct isolates of HIV-2 on CD4-negative target cells.

MAb 12G5 Reacts with Fusin

Although 12G5 was shown to react with the cell surface by FACS, efforts to immunoprecipitate or immunoblot the antigen under a variety of conditions were unsuccessful. However, following the identification of Fusin as a CD4-associated accessory factor for T cell line–tropic HIV-1 isolates (Feng et al., 1996), we evaluated 12G5 for reactivity with this protein. U87 cells that stably expressed either Fusin or the CC-chemokine receptor,

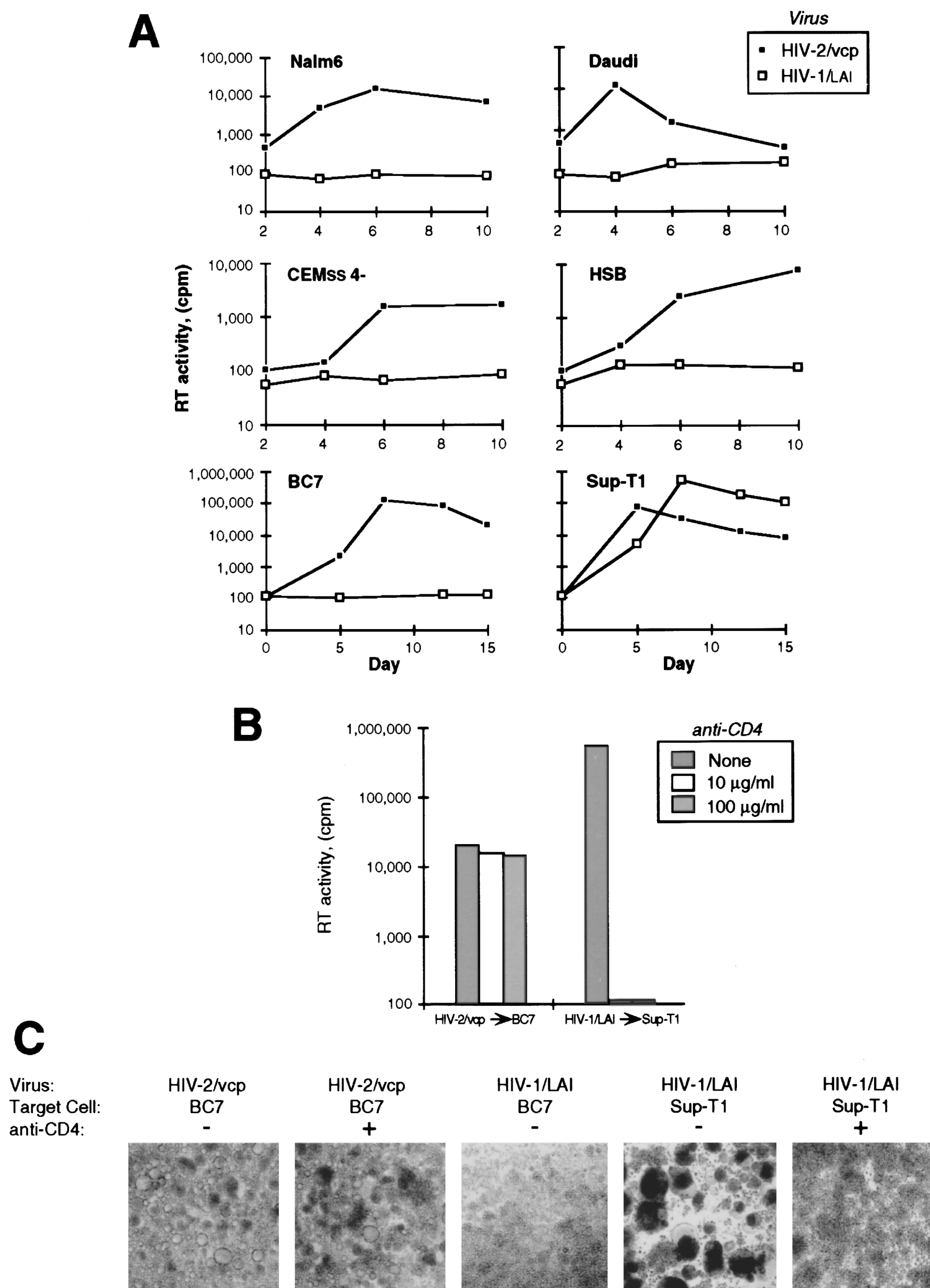


Figure 1. Infection of CD4 Negative Cells by HIV-2/vcp

(A) Cell lines were inoculated with cell-free HIV-2/vcp or HIV-1/LAI and monitored for RT activity in culture supernatant at the indicated time points. Except for Sup-T1, all cell lines were shown to be CD4-negative as determined by FACS analysis using a panel of anti-CD4 MAbs and/or by Western blot using an anti-CD4 serum.

(B) Failure of an anti-CD4 MAb to inhibit HIV-2/vcp infection of a CD4-negative line. BC7 or Sup-T1 cells were inoculated with either HIV-2/vcp or HIV-1/LAI, respectively, in the presence or absence of anti-CD4 MAb #19. Cells were maintained in the presence of the anti-CD4 MAb for 8 days at which time RT activity was determined. Similar results were seen using the anti-CD4 MAb OKT4A (not shown).

(C) Syncytium induction assays were performed on the indicated target cells by cocultivation with HIV-2/vcp-infected BC7 cells or HIV-1/LAI-infected Hut-78 cells in the presence or absence of 10 μg/ml of anti-CD4 MAb #19. Cultures were photographed after either 24 or 48 hr for assays involving HIV-1/LAI or HIV-2/vcp, respectively. As shown, extensive syncytium formation is induced by HIV-2/vcp on CD4 negative BC7 cells and is unaffected by the anti-CD4 MAb.

CCR1, were derived and evaluated by FACS for 12G5 binding. Remarkably, 12G5 reacted strongly with U87 cells that expressed Fusin while only background staining was detected on U87 cells that expressed CCR1 or cells transduced with the control vector alone (Figure 3A). Similar results were seen for human 293T cells in which Fusin was transiently expressed by transfection (not shown).

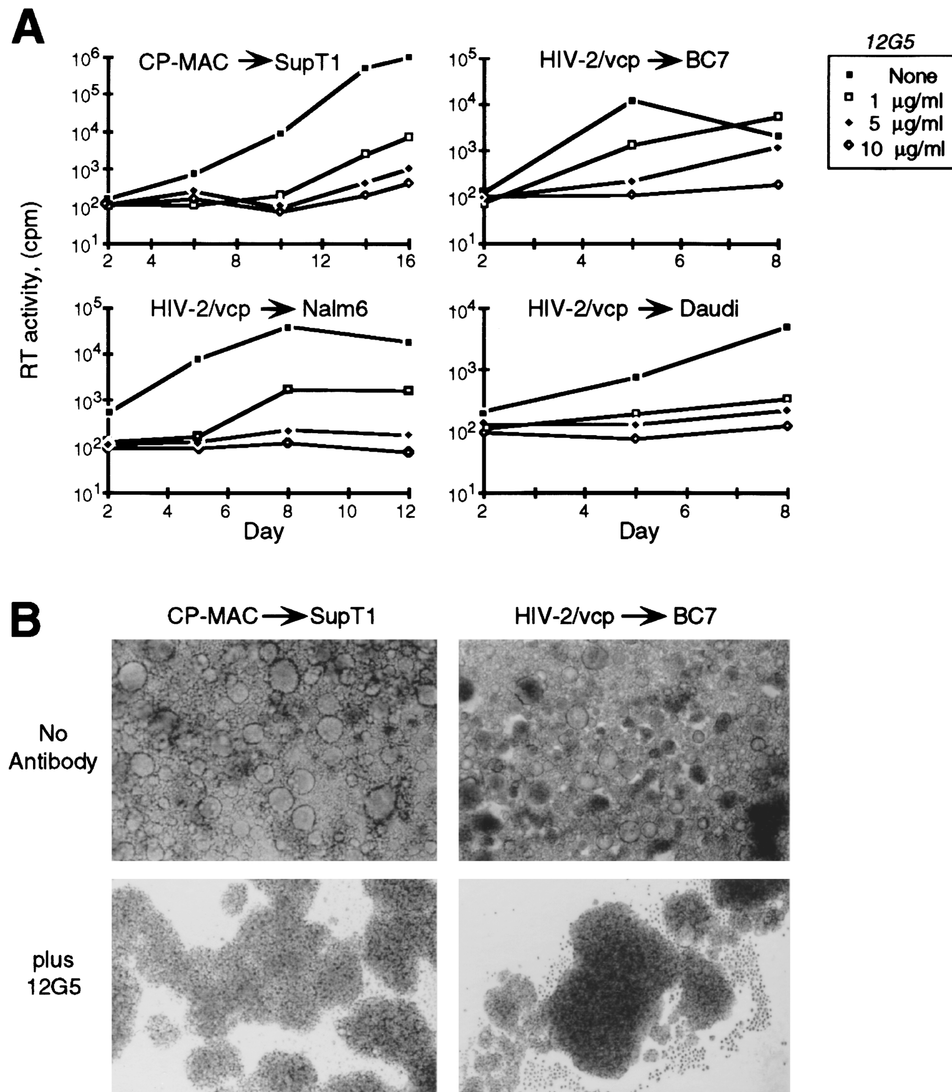


Figure 2. Inhibition of CP-MAC and HIV-2/vcp Syncytium Induction and Infection by the 12G5 MAb

(A) Sup-T1 cells or CD4-negative BC7, Nalm6, and Daudi cells were preincubated with the concentrations of 12G5 indicated, inoculated with the viruses shown, and serially monitored for RT activity in culture supernatants as described in Experimental Procedures. Dose-dependent inhibition of CP-MAC and HIV-2/vcp infection by 12G5 is shown.

(B) Sup-T1 or BC7 cells were cultured with either CP-MAC-infected Sup-T1 cells, or HIV-2/vcp-infected BC7 cells in the presence or absence of 12G5 (10 μg/ml) and photographed after 48 hr. Inhibition of syncytium formation by 12G5 is seen for both viruses.

To determine if 12G5 reacted with other chemokine receptor family members, a panel of CHO cell lines was used that stably expressed either Fusin, CC-chemokine receptors (CCR1, CCR2b, CCR3, CCR4, or CCR5), or CXC-chemokine receptors (IL8R-A or IL8R-B). The recombinant receptors in these CHO lines were expressed either untagged or tagged at the amino terminus with the influenza hemagglutinin (HA) epitope. Surface binding assays were performed using ^{125}I-labeled 12G5 and showed specific binding only to cells that expressed Fusin (Figure 3B). Scatchard analysis of 12G5 binding on CHO cells expressing HA-tagged Fusin or to RD or BC7 cells showed approximately 10^6, 4×10^5, and 5×10^4 antibody molecules bound per cell, respectively, with K_ds of 1–5 nM (not shown).

Analysis of Fusin-expressing CHO cells was also performed by immunofluorescence confocal microscopy of intact cells. As shown in Figure 3C, staining was observed on cells expressing HA-tagged Fusin while no reactivity was seen with cells expressing HA-tagged IL8R-B receptors. Expression of each protein could be detected using an HA-specific MAb, and no staining was observed with an anti-CD4 MAb used as a control (Figure 3C). Similar negative results were seen when CHO cells expressing other chemokine receptors including, IL8R-A, CCR1, CCR2b, CCR3, CCR4, and CCR5 were stained with 12G5 (not shown). Intracellular Fusin was also detectable by 12G5 on cells permeabilized with saponin (not shown). Therefore, 12G5 bound specifically to both human and nonhuman cells that expressed re-

Table 1. Reactivity of 12G5 Monoclonal Antibody with Different Cell Lines

Cell Line	Species/Origin	12G5 Reactivity	CD4
Sup-T1	Human/T cell	+	+
Hut-78	Human/T cell	+	+
CEMss	Human/T cell	+	+
Molt4 clone 8	Human/T cell	+	+
HSB	Human/T cell	+	−
BC7	Human/T cell	+	−
CEMss4$^-$	Human/T cell	+	−
CEMx174	Human/TxB hybrid	+	+
Daudi	Human/B cell	+	−
Nalm6	Human/B cell	+	−
KM3.79	Human/B cell	+	−
REH	Human/B cell	+	−
RD	Human/rhabdomyosarcoma	+	−
Hela	Human/cervical carcinoma	+	−
HOS	Human/osteosarcoma	−	−
U87	Human/astrocyte, glial	−	−
CCCS+L−	Feline/kidney	−	−
QT6-5	Quail/fibrosarcoma	−	−
CHO	Hamster/ovary	−	−
SP2	Murine/B cell	−	−

Cells were stained for FACS analysis with saturating amounts of 12G5 (10–15 μg/ml). Reactivity was compared to an isotype-matched control MAb. A panel of anti-CD4 MAbs was used to evaluate CD4 expression including OKT4, OKT4A, and #19. Plus sign indicates reactivity ≥3-fold background. CEMss4$^-$ cells are a CD4-negative variant of CEMss derived by serially panning with an anti-CD4 MAb, sorting for CD4-negative cells and cloning by limiting dilution. BC7 cells were cloned by limiting dilution from a culture of Sup-T1 cells chronically infected by HIV-1/NL4-3 and show no detectable HIV-1, as determined by p24 production, coculture assay with susceptible cells, and PCR of genomic DNA with virus-specific primers (M. J. E. and J. A. H., unpublished). Lysates of BC7 and CEMss4$^-$ exhibit no detectable CD4 protein by Western blot using a rabbit anti-CD4 antiserum.

combinant Fusin, strongly suggesting that this antibody reacts specifically with the human Fusin protein.

Fusin Serves as an Alternative Receptor for HIV-2/vcp in the Absence of CD4

Because 12G5 was able to inhibit CD4-independent infection by some HIV-2 isolates, the above findings suggested that Fusin was functioning as an alternative receptor for these viruses. In order to test this hypothesis, U87 cells that stably expressed either recombinant Fusin or CD4 were evaluated in coculture assays for their ability to form syncytia with HIV-2/vcp-infected cells. As previously noted, U87 cells exhibited no reactivity with 12G5 by FACS analysis (Table 1) and have no detectable *Fusin* mRNA (Feng et al., 1996; A. McKnight et al., submitted). As shown (Figure 4A), when Fusin-expressing U87 cells were cocultured with HIV-2/vcp-infected BC7 cells, extensive cell fusion was seen. Preincubating cells with 12G5 (10 μg/ml) completely abolished syncytium formation. No fusion was seen when similar cocultures were performed with nontransduced U87 cells or with U87 cells that expressed only recombinant CD4 (Figure 4A). Similar results were seen for murine NIH 3T3 fibroblast cells that stably expressed Fusin in the absence of CD4 (not shown).

Experiments were performed to determine if recombinant Fusin could mediate productive infection by cell-free HIV-2/vcp. Control U87 cells or U87 cells that stably expressed either Fusin or CD4 were incubated with HIV-2/vcp and monitored for infection by serial determination of reverse transcriptase activity in the culture supernatants. As shown (Figure 4B), viral replication was detectable only on U87 cells that expressed Fusin and was associated with extensive cytopathic effects including syncytium formation and cell killing (not shown).

Finally, in order to evaluate the function of the HIV-2/vcp envelope glycoprotein in the absence of other viral gene products, the HIV-2/vcp *env* gene was PCR amplified from genomic DNA, cloned and expressed in HeLa cells, and cell-to-cell fusion quantitated as previously described using a luciferase gene reporter assay (Doranz et al., 1996). Quail QT6 target cells were transfected with a luciferase reporter gene and either Fusin alone or Fusin with CD4. As shown (Figure 5), the HIV-2/vcp envelope glycoprotein fused with Fusin-expressing tar-

Table 2. Inhibition of HIV-2 Syncytium Induction by 12G5 on CD4-Negative Cell Lines[a]

HIV-2-Infected Cell[b]	Target Cell[c]	
Cell/Virus	RD	Daudi
H9/HIV-2 Rod-B	2.5	5.0
H9/HIV-2 Rod-A plus sCD4	5.0	5.0
H9/HIV-2 CBL-23 plus sCD4	5.0	5.0

[a] CD4-negative RD or Daudi cells were treated for 30 min with varying concentrations of 12G5 before adding an equal number of H9 cells chronically infected with the HIV-2 isolate indicated. Cultures were maintained at 37°C overnight before counting the number of syncytia.
[b] Where indicated, infected cells were preincubated with sCD4 (1 μg/ml) at 37°C for 60 min.
[c] Numbers indicate the minimum antibody concentration (μg/ml) that inhibited syncytium formation by >95%.

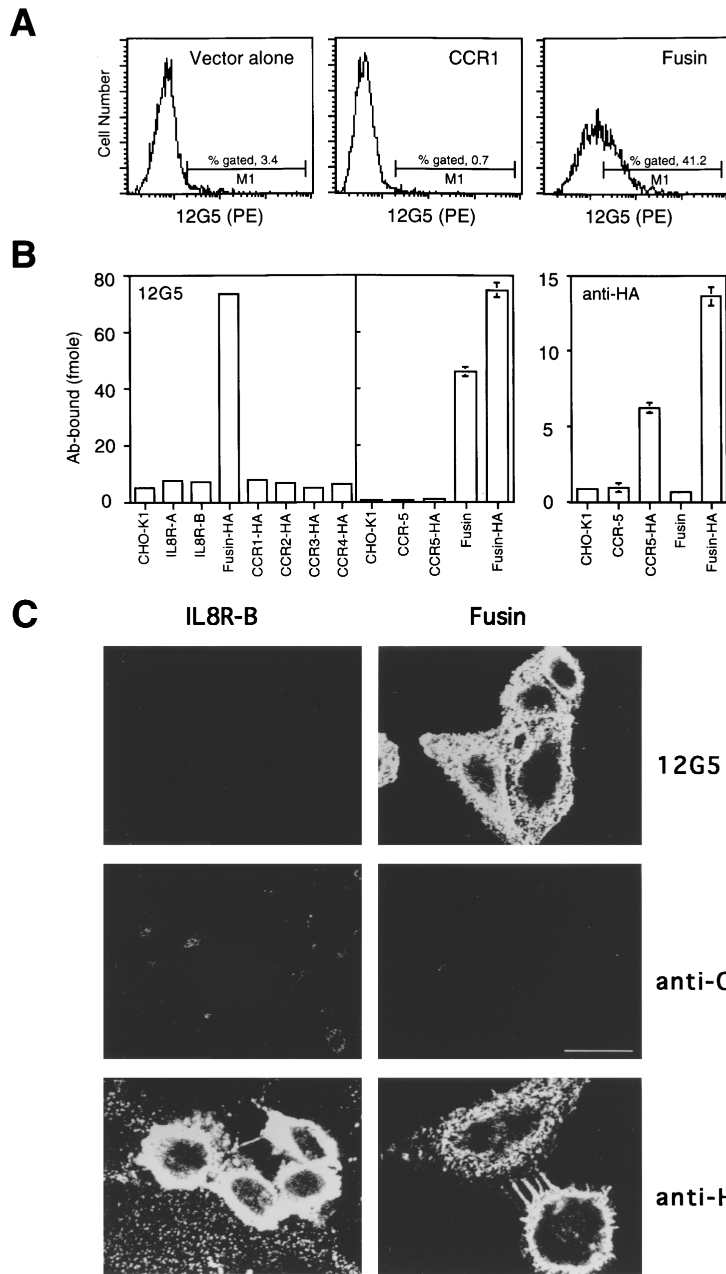


Figure 3. Reactivity of 12G5 with Fusin

(A) U87 cells stably expressing either Fusin, CCR1, or the control vector (pBABE-puro) (Deng et al., 1996) were evaluated for reactivity with 12G5 (10 μg/ml) by FACS. The region for positivity, designated M1, was defined using a control MAb and the percentage of cells falling within this window for each sample indicated.

(B) Control CHO cells or cells stably expressing HA-tagged or untagged Fusin or the other chemokine receptors indicated were evaluated for reactivity to [^{125}I]12G5 or [^{125}I]12CA5 (anti-HA), using protocols described previously (Pelchen-Matthews et al., 1989). Scatchard-type analysis indicated that the K_d for 12G5 binding on CHO-Fusin cells was 1–5 nM; [^{125}I]12G5 binding was competed close to background levels by 100 nM 12G5 but was not influenced by the anti-HA antibody 12CA5 (data not shown).

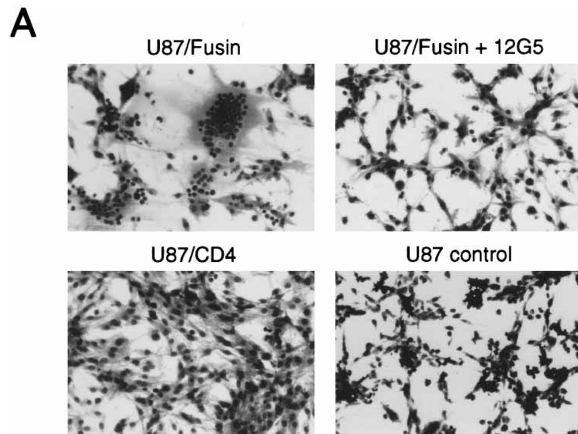
(C) Immunofluorescence confocal microscopy of CHO cells stably expressing Fusin or chemokine receptors using 12G5. CHO-K1 cells expressing HA-tagged Fusin or the human IL8R-B receptor were stained with 12G5, the anti-human CD4 MAb Q4120, or an antibody against the HA-tag (12CA5). Scale bar = 20 μm.

get cells in the presence and absence of CD4. In contrast a comparably expressed BH8 envelope glycoprotein derived from a T cell line–tropic HIV-1 induced fusion on Fusin-expressing cells only in the presence of CD4. No fusion was seen when HIV-2/vcp *env*-expressing cells were cultured with QT6 cells transfected with the IL8R-B receptor or the expression vector alone (Figure 5). Taken together, these results show that Fusin can serve as an alternative receptor for some isolates of HIV-2 in the absence of CD4 on human as well as nonhuman target cells. These findings also demonstrate that for HIV-2/vcp, CD4 alone is not sufficient to permit viral entry and indicate that the utilization of Fusin is an obligatory step for this virus during infection.

Down-Regulation of Surface Fusin by HIV-2/vcp Infection

Cellular receptors for enveloped viruses are characteristically down-regulated from the cell surface following productive infection, rendering infected cells resistant to superinfection by viruses that utilize the same receptor (Weiss, 1993). Indeed, the observation that CD4 was selectively down-regulated on HIV-1-infected cells provided the initial evidence that CD4 was a receptor for this virus (McDougal et al., 1986). Down-regulation of CD4 and other viral receptors by envelope glycoproteins has been attributed to the formation of intracellular complexes between envelope molecules and the cellular receptors and/or to the blocking of binding sites on

A



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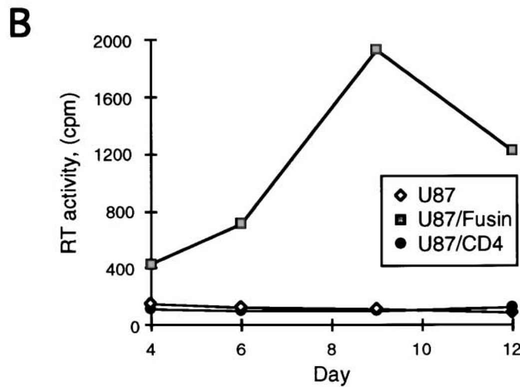

Figure 4. Recombinant Fusin Is Sufficient to Render U87 Cells Susceptible to HIV-2/vcp Syncytium Induction and Infection

(A) Syncytium induction assays. U87 cells stably expressing either Fusin or CD4, or untransduced cells (control) were cocultured with HIV-2/vcp-infected BC7 cells and photographed for syncytium formation as described in Experimental Procedures. Large syncytia are evident only in U87 cells expressing Fusin, and this was completely inhibited by preincubating cells with 12G5 (10 μg/ml). (B) Infection assays. Cells were inoculated with cell-free HIV-2/vcp (1,000 $TCID_{50}$ units) and serial determination of RT activity performed at the indicated time points. Infection is only seen for Fusin-expressing cells. Extensive syncytium formation and cell killing were also observed in this culture that correlated with the time of RT production (data not shown).

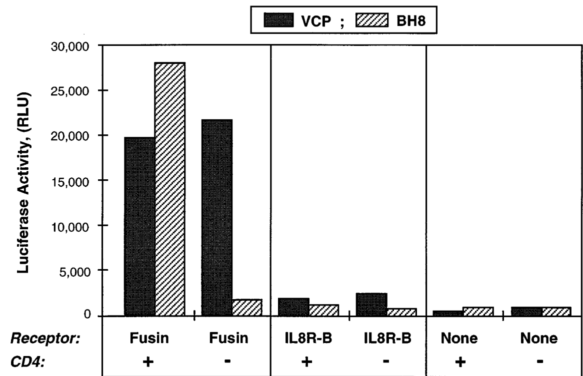

Figure 5. Induction of Cell Fusion by the HIV-2/vcp Envelope Glycoprotein in a Gene Reporter Fusion Assay

HeLa effector cells were transfected with pCR3.1 expressing either HIV-2/vcp *env* or BH8 HIV-1 *env* clones and infected with vaccinia. QT6 target cells were transfected with constructs expressing Fusin, IL8R-B or the pCR3.1 expression vector alone, and a plasmid containing the luciferase gene driven by a T7 promoter (Promega). Where indicated target cells were also infected with pT4, which constitutively expresses CD4 from the CMV promoter. Luciferase activity as an indication of cell fusion is indicated in terms of relative light units (RLU) as described previously (Doranz et al., 1996).

receptors that are expressed on the cell surface (Hoxie et al., 1986; Crise et al., 1990; Schneider-Schaulies et al., 1995). Given the evidence that Fusin was serving as a primary receptor for HIV-2/vcp, we utilized 12G5, to determine if this molecule was down-regulated during HIV-2/vcp infection.

BC7, Daudi, and Nalm6 cells that were either uninfected or chronically infected by HIV-2/vcp were evaluated for Fusin expression by FACS analysis with 12G5. As shown in Figure 6, on HIV-2/vcp-infected cells, 12G5 binding was reduced 98%, 79%, and 87% compared to uninfected cells. In contrast, no reduction was seen in expression of HLA class I or transferrin receptors using specific MAbs (not shown). Thus, for several CD4-negative lymphoid cells, HIV-2/vcp infection produced a marked and selective reduction in 12G5 binding. Although this experiment could not distinguish between the loss of surface Fusin and blocking of the 12G5 epitope, this result is entirely consistent with other evidence noted above that Fusin is serving as a primary viral receptor for HIV-2/vcp. As expected, CD4 was down-regulated 95% and 98% on HIV-2/vcp and CP-MAC-infected Sup-T1 cells, respectively (not shown). Interestingly, although Fusin was also down-regulated 75% on HIV-2/vcp-infected Sup-T1 cells, no reduction in 12G5 reactivity was seen on Sup-T1 cells that were infected by CP-MAC (Figure 6) or HIV-1/LAI (not shown). Because CP-MAC (LaBranche et al., 1994) and HIV-1/LAI are dependent on CD4, this finding indicates that Fusin down-regulation occurs when it is utilized as a primary receptor in the absence of CD4 but not as a CD4-associated coreceptor (see Discussion).

Discussion

In this report we demonstrate that Fusin, the recently described cofactor for T cell line–adapted isolates of HIV-1, is able to function as an alternative receptor for some isolates of HIV-2 in the absence of CD4. Two genetically distinct isolates of HIV-2 that exhibited CD4-independent infection of human lymphoid and nonlymphoid cells (HIV-2/vcp and HIV-2/Rod-B) were inhibited in virus neutralization and/or syncytium induction assays by 12G5, a MAb shown to be specific for Fusin. In addition, U87 cells that were resistant to HIV-2/vcp became fully permissive for syncytium formation and infection following transduction with recombinant Fusin in the absence of CD4. This effect was completely dependent on the viral envelope glycoprotein, since a cloned HIV-2/vcp *env* gene mediated syncytium formation on QT6 cells when Fusin was expressed without

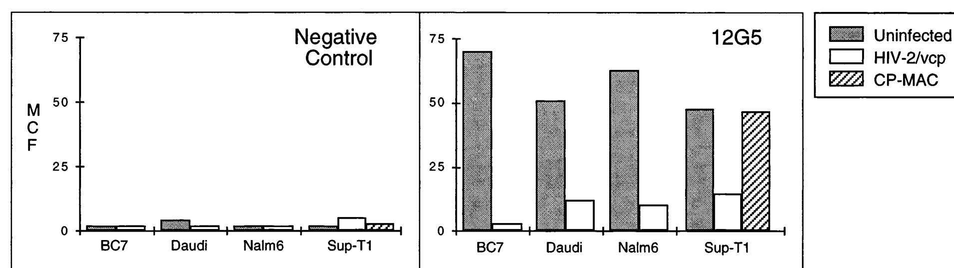

Figure 6. Downregulation of Fusin Expression by HIV-2/vcp Infection

Cell lines that were either uninfected or infected by the viruses indicated were evaluated for surface reactivity by FACS either with an isotype-matched control MAb or 12G5 (10 μg/ml), and the mean channel fluorescence intensity (MCF) shown for each cell type. Cells were 100% infected, as determined by immunofluorescence microscopy using an HIV-2/SIVmac p27*gag* -specific MAb. Loss of 12G5 reactivity is seen on HIV-2/vcp-infected but not CP-MAC-infected cells. The data are representative of three experiments from two separate infections.

CD4. Finally, as is the case for many viral receptors, HIV-2/vcp infection produced a selective down-regulation of Fusin from the cell surface.

Although several members of the chemokine receptor family have been shown to function in association with CD4 to permit viral entry (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996; Feng et al., 1996), the precise mechanism of action of these proteins as coreceptors has not been established. One recent report mapped a determinant for coreceptor utilization to the hypervariable V3 loop of the gp120 surface envelope glycoprotein (Choe et al., 1996). Additional studies using chimeric receptors between CCR5 and CCR2b have identified regions on CCR5 that are required for this protein to function as a CD4 coreceptor for M- and dual-tropic HIV-1 isolates (Rucker et al., 1996). These findings are consistent with the view that an initial interaction of the envelope glycoprotein with CD4 produces a conformational change that facilitates a secondary interaction with one or more 7tm proteins (Deng et al., 1996; Doranz et al., 1996). However, it is also possible that 7tm proteins facilitate viral entry indirectly by affecting the cell membrane and/or CD4 without contacting the viral envelope molecule (Berson et al., 1996; Choe et al., 1996). Our finding, that at least one member of this family can serve as a primary receptor in the absence of CD4 for a subset of HIV-2 isolates, strongly suggests that these proteins interact directly with the viral envelope glycoprotein during entry. Moreover, the finding that a chemokine receptor, unlike CD4, can be both necessary and sufficient for viral entry, raises the intriguing possibility that these 7tm proteins may be primordial receptors for the primate lentiviruses. Thus, the role of CD4 may simply be to direct the viral envelope glycoprotein onto these molecules where the subsequent fusion reaction then occurs.

It remains to be determined what changes in the envelope glycoprotein enable the HIV-2 variants that we have analyzed to bypass a requirement for CD4. Recent studies of HIV-2/Rod-B have indicated that mutations in both gp120 as well as TM are required for this virus to infect CD4-negative cells (Reeves and Schulz, 1996). These mutations could increase exposure of a binding site for a 7tm protein and/or increase the affinity of this interaction. Interestingly, although Fusin is down-regulated by HIV-2/vcp in the context of CD4-independent infection, it is not down-regulated by CP-MAC, a virus that still requires CD4 for infection and cell fusion (Figure 6). Recent studies have also shown that HIV-1/LAI, which requires Fusin in association with CD4 to infect cells, induces minimal (<10%) down-regulation of surface Fusin on chronically infected cells (J. A. H., unpublished data). If the ability of HIV-2/vcp to utilize Fusin as an alternative receptor corresponds to a higher affinity interaction with the envelope glycoprotein, it follows that the transport of Fusin to the cell surface during synthesis could be inhibited in infected cells as has been shown to occur for CD4 (Crise et al., 1990; Jabbar and Nayak, 1990; Koga et al., 1990). In contrast, for viruses that might bind more weakly to Fusin (i.e., in a CD4-dependent manner) an intracellular interaction could either fail to occur or not be sufficiently stable to interfere with the surface expression of Fusin. Viruses such as HIV-2/vcp and HIV-2/Rod-B will be particularly useful in biochemical assays to evaluate direct interactions of their envelope glycoproteins with Fusin and perhaps other members of the chemokine receptor family.

Although in general, HIV and SIV isolates are CD4-dependent, our finding that two genetically distinct isolates of HIV-2 can infect Fusin-expressing cells independent of CD4 indicates that this property is not restricted to a single laboratory variant. Indeed, as initially reported, HIV-2s with only a minimal passage history have exhibited CD4-independence in vitro (Clapham et al., 1992). While the relevance of this finding for pathogenesis, if any, remains to be determined, it is possible that the ability of some HIV isolates to utilize a 7tm molecule as a primary receptor could have implications for pathogenesis. Studies of tissues from HIV-infected humans (Wiley et al., 1986; Bagasra et al., 1996; Livingstone et al., 1996; Moses et al., 1993) and SIV-infected nonhuman primates in advanced stages of disease (Mankowski et al., 1994; Dean et al., 1996) have shown productive infection of cells that are apparently CD4-negative. Although it is possible that these cells do express a low level of CD4, our study raises the possibility that HIV could evolve in vivo to bypass CD4 and utilize at least some chemokine receptors directly to infect cells. Given the expanding number of molecules that are part of the chemokine receptor family (Power and Wells, 1996), and the ability of HIV isolates (Feng et al., 1996; Alkhatib et al., 1996; Dragic et al., 1996; Deng et al., 1996; Doranz

et al., 1996; Choe et al., 1996) to interact with different members of this family, it is possible that direct, CD4-independent interactions between envelope molecules and 7tm proteins could occur in vivo on a variety of cell types in the immune, hematopoietic, or nervous systems. By broadening the viral host range and/or causing alterations in cellular functions, such interactions could be highly relevant to clinical complications of AIDS.

Understanding the mechanisms through which HIV and SIV utilize 7tm molecules, either directly or in association with CD4, will provide considerable insight into the molecular basis for differences in tropism among these viruses. However, it is clear that coreceptor utilization is structurally complex and that a single envelope glycoprotein can interact with genetically divergent members of the chemokine receptor family (Choe et al., 1996; Doranz et al., 1996). Moreover, recent studies with 12G5 have shown that the ability of this antibody to inhibit T-tropic isolates of HIV-1 is highly dependent on the viral isolate and the target cell (A. McKnight et al., submitted). This finding has suggested (1) that other cofactors in addition to Fusin are involved on particular cell types; (2) that post translational modifications occur on Fusin that are relevant to its interaction with the envelope glycoprotein and 12G5; and/or (3) that viruses may utilize different epitopes on Fusin, only some of which are blocked by 12G5. Even though CP-MAC infection of Sup-T1 cells is inhibited by 12G5, this virus is unable to infect or fuse with several T cell lines that coexpress high levels of CD4 and Fusin, including CEMx174 and HUT-78 cells (LaBranche et al., 1994, 1995). Thus, still other molecules may be required as cofactors and determinants of tropism for some HIV and SIV isolates.

The availability of 12G5, which reacts with cell surface as well as intracellular Fusin, provides an important reagent for basic studies of this receptor at the cellular level including (1) its association with CD4 and other surface molecules, (2) its distribution within tissues and among subsets of hematopoietic cells, and (3) its participation in cellular signal transduction pathways. We recently utilized 12G5 to demonstrate rapid internalization of Fusin on lymphoid cells in response to phorbol esters (J. A. H. and M. M., unpublished data), a property originally predicted for CD4-associated cellular cofactors of HIV-1 (Golding et al., 1994, 1995). Finally, because interaction of 7tm G protein–coupled receptors with their natural ligands frequently induces their internalization (von Zastrow and Kobilka, 1992; Hoxie et al., 1993; Grady et al., 1995), 12G5 may also be useful in evaluating the effects of physiologic ligands and potential agonists for this receptor. Future studies with this antibody should provide important insights into the structure of Fusin and help to identify domains involved in its physiologic role as a signal transduction molecule and in its pathologic role as a receptor for HIV.

Experimental Procedures

Cells

CD4-positive human T lymphoid cell lines, Sup-T1, Hut-78, H9, CEM, Molt4-clone8, and the TxB cell hybrid line CEMx174 have been described previously (Hoxie et al., 1986, 1988; LaBranche et al., 1994). CD4-negative T lymphoid lines HSB and CEMss4$^-$ were provided by David Weiner (University of Pennsylvania) and Carolyn Doyle (Duke University), respectively, and BC7 was derived from Sup-T1 cells, as described in Table 1. Human B cell lines, Nalm6, KM3.79, and REH were provided by Lyn Healy and Mel Greaves. Hela, RD, and Daudi cells have been described previously (Clapham et al., 1991, 1992). 293T and CCCS+L− cells were provided by Michael Malim (University of Pennsylvania); QT6-5 Japanese quail fibrosarcoma cells were provided by Paul Bates (University of Pennsylvania), and murine myeloma SP2 cells and the W6/32 hybridoma cell line were obtained from the American Type Culture Collection. U87 cells stably expressing recombinant Fusin, CCR1, or CD4 were derived as described previously (Deng et al., 1996). Murine NIH 3T3 cells expressing either Fusin alone or Fusin and human CD4 were provided by D. Littman (New York University) (Deng et al., 1996). Chinese Hamster Ovary cells (CHO-K1) expressing HA-tagged or untagged human chemokine receptors were derived using cDNAs derived by RT–PCR and cloned into pcDNA1neo for expression in mammalian cells (Power et al., 1995). Stable CHO lines expressing the chemokine receptors were derived by screening for reactivity to the anti-HA MAb, 12CA5. The receptor selectivity of the HA-tagged receptor lines was tested by radioligand binding assays using membranes purified from the respective cells. No significant differences in ligand binding properties were seen between tagged and nontagged receptors (T. N. C. W., unpublished data).

Viruses

An uncloned viral stock of HIV-2/NIH-z was provided by R. Gallo. HIV-2/vcp was derived from HIV-2/NIH-z (Zagury et al., 1988) by passaging virus first onto CP-MAC infected Sup-T1 cells that had completely down-regulated their surface expression of CD4 (LaBranche et al., 1994), and then onto BC7 cells. Sequence analysis of the HIV-2/vcp *env* gene amplified from genomic DNA showed no evidence of recombination in *env* with CP-MAC. CP-MAC was derived as previously described from the SIVmac molecular clone, BK28 (LaBranche et al., 1994). Additional variants of HIV-2 described for their ability to infect CD4-negative cells included HIV-2/CBL-23, HIV-2/Rod-A, and HIV-2/Rod-B (Clapham et al., 1992).

Constructs

The HIV-2/vcp *env* gene was PCR amplified from genomic DNA from HIV-2/vcp-infected HSB cells using the primer pair 5′-GGCTCA TCCGGTCGACGAATCAGACAAGTGAGTATGAAGGGTAGTAAG-3′ and 5′-CTGCTGATATCGCTGTCCCTCACAGGAGGGCGAG-3′, and cloned into the eukaryotic expression vector pCR3.1 (Invitrogen). The BH8 *env* clone was provided by Dr. Pat Earl (NIH). Fusin and CD4 were expressed as previously described (Berson et al., 1996; Doranz et al., 1996). Frank Jirik (University of British Columbia) provided the IL8 receptor clone.

Antibodies

12G5 was produced by inoculating Balb/c mice with 10^7 living CP-MAC-infected Sup-T1 cells intraperitoneally for 3 weekly injections, and fusion protocols were performed as described previously (Brass et al., 1994). Hybridomas were screened in 96-well plates for the ability to inhibit CP-MAC-induced syncytium induction on Sup-T1 cells and cloned by limiting dilution. Antibody was purified from ascites using HiTrap Protein G (Pharmacia Biotech). The anti-CD4 MAb #19 was produced using a similar protocol in which the immunizing cell type was uninfected Sup-T1 cells (J. A. H. and M. A., unpublished data). Specificity of this antibody for CD4 was determined by its ability to detect the 55 kDa CD4 protein by Western blot from lysates of CD4-positive cells (data not shown). The anti-CD4 MAbs OKT4A and OKT4 were purchased from Ortho Pharmaceuticals, and 12CA5 was purchased from Boehringer-Mannheim. D47 is an IgG_{2a} MAb reactive with the HIV-1/LAI gp120. Rabbit anti-human CD4 serum was provided by R. Sweet (SmithKline Beecham).

Viral Infection and Neutralization Assays

Viral infection assays on lymphoid cell lines and U87 cells expressing recombinant Fusin or CD4, were performed as previously described by inoculating cultures with 1000 $TCID_{50}$ units of either HIV-1/LAI or

HIV-2/vcp. Input virus was washed out after 24 hr and cultures were monitored for infection by serial determinations of reverse transcriptase activity and visual inspection for syncytium formation. For neutralization assays, target cells were preincubated with varying concentrations of either 12G5 or anti-CD4 MAbs for 30 min at 37°C followed by the addition of 100 $TCID_{50}$ units of virus, as titered on each target cell. Input virus was removed after 24 hr and cultures were monitored for infection as above. Cells were maintained in the presence of antibody for the duration of the experiment.

Cell Fusion Assays

Syncytium induction assays were performed as previously described (LaBranche et al., 1994). In brief, HIV- or SIV-infected cells were added to target cells growing as suspension cultures or adherent cells at a ratio of approximately 1 to 5. After 24 or 48 hr, samples were either inspected visually by phase contrast microscopy or fixed and stained with Wright stain and 10% Giemsa In selected experiments, MAbs were preincubated with target cells for 30 min at 37°C. For experiments involving HIV-2/Rod-A or HIV-2/CBL-23, which require priming with soluble sCD4 to infect CD4-negative cells, H9 cells chronically infected with these viruses were preincubated with 5 μg/ml of soluble CD4 prior to cocultivation with target cells as previously described (Clapham et al., 1992). Cell fusion induced by cloned *env* genes was quantitated using a luciferase gene reporter assay described previously (Doranz et al., 1996). In brief, HeLa effector cells were transfected with HIV *env*-containing constructs and infected with recombinant vaccinia virus encoding the T7 polymerase gene (provided by B. Moss, NIH). Target cells were quail QT6 cells that were transfected with a plasmid containing the luciferase gene driven by a T7 promoter (Promega). Selected target cells were also transfected with pT4 which constitutively expresses CD4 from the CMV promoter. Effectors and target cells were mixed and allowed to fuse for 8 hr. Cells were then washed with PBS and lysed in 150 μl reporter lysis buffer (Promega) and assayed for luciferase activity according to the instructions of the manufacturer.

Flow Cytometry

Surface expression of cell surface antigens was performed by FACS analysis using a Becton-Dickinson FACScan flow cytometer as described previously (LaBranche et al., 1994). For experiments involving adherent cells, cells were dislodged from plates by treatment with PBS/1 mM EDTA. All HIV- or SIV-infected cell samples were fixed at 4° for 24 hr in 4% paraformaldehyde prior to analysis.

Iodinated Antibody Binding Assays

Binding assays with iodinated 12G5 and iodinated 12CA5 were performed essentially as described (Pelchen-Matthews et al., 1989). 12G5 and 12CA5 were labeled with ^{125}I using Bolton and Hunter reagent (Amersham, UK) to a specific activity of 390 and 550 Ci per mmol, respectively. Cells were cultured in 24-well plates and incubated with 1 nM ^{125}I-12G5 and 2 nM 12CA5 for 2–4 hr at 4°C, then washed and the amount of bound antibody determined by γ-counting. Background levels of binding were determined by performing assays in the presence of 100 nM unlabeled antibody.

Immunofluorescence Microscopy

CHO cells either stably or transiently expressing Fusin or other chemokine receptors were cultured on glass coverslips and fixed in 3% paraformaldehyde. Cells were washed, quenched in 50 mM NH_4Cl and 0.2% fetal calf serum, and stained with the respective antibodies for 1 hr at room temperature. Cells were then washed, stained with TRITC-conjugated goat anti-mouse IgG for 1 hr, washed again and mounted in Moviol. Cells were photographed using a Nikon Optiphot-2 microscope equipped with an MRC Bio-Rad 1024 laser scanning attachment.

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