Human immunodeficiency virus type 1 (HIV-1) superinfection of a cell clone converting it from production of defective to infectious HIV-1 is mediated predominantly by CD4 regions other than the major binding site for HIV-1 glycoproteins

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A cell clone, L-2, which produces non-infectious doughnut-shaped human immunodeficiency virus type 1 (HIV-1) particles, was permissive for HIV-1 superinfection, which resulted in the production of infectious particles. The superinfection showed slow kinetics compared with primary HIV-1 infection of M10 cells, the parent of the L-2 cell clone. Inhibition studies on the superinfection of L-2 cells using several CD4-related reagents showed that the CD4 molecule was an essential component of the receptor for superinfection. Strong inhibitory effects were obtained using CD4 peptides such as CD4(68-130), which includes a portion homologous to the immunoglobulin third complementarity-determining region (CDR3), as well as recombinant soluble CD4. In contrast, a CD4(45-60) peptide, which includes most of the CDR2-related region, was not effective, although the Leu-3a monoclonal antibody (MAb), which recognizes a site near the CDR2-related region, did slightly, but significantly, delay the superinfection kinetics. Comparative flow cytometry of L-2 and M10 cells revealed that the cell surface of L-2 cells despite expressing HIV-1 env protein, reacted slightly with OKT4 or anti-CD4(68-130) MAb, but not with Leu-3a or OKT4A MAb. In contrast, no reaction was detected with any of these anti-CD4 MAb's on the surface of another HIV-1 superinfection-resistant cell clone, MOLT-4IIIB-14, which expresses HIV-1 env proteins but does not produce infectious HIV-1 particles. These results strongly suggest that expression of the CD4 major receptor site for primary HIV-1 infection is preferentially decreased on the surface of L-2 cells, but that the OKT4 epitope and the nearby region corresponding to immunoglobulin CDR3 remain exposed on the cell surface. Consequently, the CD4 CDR3-related region could play a major role as the receptor for the superinfection reported here.

Introduction

Human immunodeficiency virus type 1 (HIV-1) is a member of the lentivirus family of retroviruses, and is aetiologically associated with the development of AIDS (Barré-Sinoussi et al., 1983; Popovic et al., 1984b). HIV-1 predominantly utilizes the CD4 glycoprotein to gain entry into target cells (Dalgleish et al., 1984; Klatzmann et al., 1984; Popovic et al., 1984a), although other routes of entry independent of CD4 have been described for several cell systems (Harouse et al., 1989; Cao et al., 1990). Infection of cells with HIV-1 in vitro generally induces c.p.e. characterized by syncytium formation (Popovic et al., 1984b). For both HIV-1 infection and HIV-1-induced syncytium formation interaction of HIV-1 env gp120 with CD4 is the initial step (Lifson et al., 1986; McDougal et al., 1986; Sodroski et al., 1986). The extracellular portion of CD4 can be divided into four immunoglobulin-related domains (domains 1 to 4) (Clark et al., 1987; Maddon et al., 1987). In addition, three regions analogous to the immunoglobulin complementarity-determining region (CDR1 to 3) of an antibody-combining site can be found within domain 1 (Sattentau et al., 1989). Mutational analysis of recombinant soluble CD4 (sCD4) has demonstrated that the CDR2-related region is important for binding to gp120 (Clayton et al., 1988; Landau et al., 1988; Mizukami et al., 1988; Arthos et al., 1989; Sattentau et al., 1989), and several monoclonal antibodies (MAbs) recognizing sites near the CDR2-like region, such as Leu-3a and OKT4A (Landau et al., 1988; Mizukami et al., 1988; Peterson & Seed, 1988; Sattentau et al., 1989), block both HIV-1 infection and syncytium formation (Dalgleish et al., 1984; Sattentau et al., 1986; Sodroski et al., 1986).
addition, blocking effects of synthetic CD4 peptides such as CD4(68–130) have shown that the CDR3-related region is also involved in both HIV-1 infection and syncytium formation (Lifson et al., 1988; Hayashi et al., 1989; Nara et al., 1989; Ohki et al., 1990).

During HIV-1 infection, the expression of cell surface CD4 is greatly reduced (Hoxie et al., 1986; Stevenson et al., 1987; Salmon et al., 1988). The decrease in the amount of the CD4 receptor on the surface of infected cells appears to be mediated by the down-regulation of CD4 transcription (Hoxie et al., 1986; Salmon et al., 1988) or, more probably, by post-transcriptional events that interfere with transport of CD4 molecules to the host cell surface (Stevenson et al., 1987; Salmon et al., 1988). In fact, several reports support the latter possibility and show that CD4 molecules are still synthesized in cells transfected with the env gene, but that they form a complex with the env protein in the cytoplasm and lose the capacity to be transported to the cell surface (Kawamura et al., 1989; Buonocore & Rose, 1990; Crise et al., 1990; Koga et al., 1990). In addition, it has recently been reported that the product of an HIV-1 regulatory gene, nef, could also play a role in CD4 down-modulation (Garcia & Miller, 1991). Thus, by a number of routes, HIV-1 infection induces a decrease in CD4 expression on the infected cell surface, and therefore cells already infected with HIV-1 should be resistant to HIV-1 superinfection, as has been observed with other retroviruses (Ishizaki & Vogt, 1966; Weiss, 1969). In accordance with this it has been reported that all HIV-1 isolates examined cross-interfere and that HIV-2 interferes with HIV-1 superinfection (Hart & Cloyd, 1990). However, HIV-1 interference in HIV-2 superinfection is only partial despite both viruses using the CD4 molecule as receptor (Hart & Cloyd, 1990). In addition, it has been shown that HIV-1 env protein expressed from a retrovirus vector induces partial but not complete interference with HIV-1 infection (Stevenson et al., 1988). Thus, the factors that determine the level of interference observed between any two HIV-1 strains remain to be determined.

We have previously isolated a cell clone, termed L-2, which continuously produces non-infectious doughnut-shaped HIV-1 particles (Goto et al., 1990). The L-2 cell clone was isolated by limiting dilution of cells which had survived drastic cytolysis induced by HIV-1 infection of a clonal cell line (M10) of MT-4 cells (Miyoshi et al., 1982). Recently, we have shown that L-2 cells are permissive for HIV-1 superinfection and become producers of infectious HIV-1 particles as a result of complementation and/or recombination events in the superinfected cell (Yunoki et al., 1991). Here we have examined the identity of the receptor required for superinfection in the L-2 cell system.

Methods

Cell and virus culture. The L-2 cell clone was isolated by limiting dilution of a clonal derivative, M10, of the human T-lymphotropic virus type 1 (HTLV-I) transformed MT-4 cell line (Miyoshi et al., 1982) infected with the LAI strain of HIV-1 (Barré-Sinoussi et al., 1983) and cultured for several months as described previously (Goto et al., 1990; Yunoki et al., 1991). A further cell clone, MOLT-#8/IIIB-14, was isolated by limiting dilution of MOLT-4 clone no. 8 (MOLT-#8) (Kikukawa et al., 1986), which was inoculated with the HTLV-IIIB strain of HIV-1 (Popovic et al., 1984b) at a m.o.i. of about 1 for 1 h. The conditioned medium of MOLT-4/HTLV-IIIB cells was used as an inoculum. The MOLT-4/HTLV-IIIB cells were obtained by serial passage of acute lymphocytic leukaemia-derived MOLT-4 cells (Minowada et al., 1972) after infection with HTLV-IIIB. These cells were maintained in complete medium [RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS)].

CD4 peptide synthesis. Three CD4 peptides were synthesized, based on the amino acid sequence derived from the nucleotide sequence (Maddon et al., 1985; Litman et al., 1988), by the manual solid phase method based on Fmoc chemistry as described previously (Hayashi et al., 1989). These were CD4(68–130) and CD4(66–92), including the whole and partial CDR3-related region, respectively, and CD4(45–60), including most of the CDR2-related region. The sulphydryl groups of Cys residues were protected with an acetylimidomethyl or a benzyl group. Each peptide was used after purification by gel filtration and HPLC. The purity of synthetic peptides was assessed by amino acid analysis and HPLC (70 to 95%).

Antibodies. Anti-HIV-1 gag p18 (V17) (Ikuta et al., 1989) and env gp120 [0.5f] (Matsushita et al., 1988) MAb were used, as were anti-CD4 MAb Leu-3a (Becton Dickinson), OKT4A and OKT4 (Ortho Diagnostic Systems). MAb Leu-3a and OKT4A recognize sites near the CDR2-related region in the V1 domain (Landau et al., 1988; Mizukami et al., 1988; Peterson & Seed, 1988; Sattenau et al., 1989), whereas OKT4 recognizes the V3 and V4 domains of CD4 (Berger et al., 1988). MAb #35, recognizing CD4 amino acid residues 72 to 84, was also used and was prepared by using CD4(68–130) peptide as an immunogen (Ohki et al., 1992).

HIV-1 infectivity titeration. The infectivity of HIV-1 in the conditioned medium was titrated on M10 cells in 96-well microplates (Corning) as described previously (Ikuta et al., 1987). After culture for 4 days, HIV-1-specific antigens in the infected cells were identified by an indirect immunofluorescence (IF) test with MAb V17, and the titres were expressed as TCID50/ml.

Superinfection. L-2 and MOLT-#8/IIIB-14 cells at a density of 5 x 10^5/ml in complete medium were inoculated with HTLV-IIIB at different multiplicities. After incubation for 24 h at 37°C, the cells were washed with RPMI-1640 medium which was then replaced with the same volume of complete medium. Thereafter, the cell density was adjusted to 5 x 10^5/ml in fresh complete medium every 4 days, and the cells and conditioned media were subjected to an IF test and a reverse transcriptase (RT) assay, respectively.

IF test. The IF test was carried out using cell smears fixed with cold acetone. The cells were incubated with specific antibodies for 15 min at room temperature, and then reacted with a 40-fold dilution of fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (Dakopatts) and incubated for 15 min at room temperature.

RT assay. RT activity in conditioned medium was assayed as described previously (Ogawa et al., 1989). Briefly, the medium was mixed with a reaction mixture containing poly(A)-oligo(dT) and [α-32P]dUTP (800 Ci/mmol; New England Nuclear). After incubation
Assay of superinfection inhibition by several CD4-related reagents. Synthetic CD4 peptides, anti-CD4 MAbs and sCD4 were tested for their inhibitory effect on the superinfection of L-2 cells by HIV-1. sCD4 was expressed by a baculovirus vector as described previously (Morikawa et al., 1990). Before superinfection, L-2 cells were treated with different amounts of the anti-CD4 MAbs for 30 min at 37°C, or the conditioned medium containing HTLV-IIIB was treated with different amounts of synthetic CD4 peptides or sCD4 for 30 min at 37°C. These mixtures were then reacted with untreated conditioned medium containing HTLV-IIIB or L-2 cells. After incubation for an additional 24 h at 37°C at a cell density of 5 x 10^5/ml, the cells were washed with complete medium and cultured in complete medium without these CD4-related reagents. Thereafter, the cell density was adjusted to 5 x 10^6 cells/ml in fresh complete medium every 4 days as described above. As a control, L-2 cells were infected with HIV-1 in the presence of 3'-azido-3'-deoxythymidine (AZT) at a final concentration of 10 µg/ml, and infected cells were also cultured in the presence of AZT.

Detection of cell surface CD4 antigen. The level of expression of the cell surface CD4 antigen was determined by flow cytometry using several anti-CD4 MAbs. As a negative control, the same concentration of normal mouse IgG was used. The cell pellet (1 x 10^6 cells) was suspended in medium containing MAbs and incubated for 2 h at 4°C, and washed twice with PBS pH 7.2 containing 2% FBS. The cells were then incubated with a 20-fold dilution of FITC-conjugated rabbit anti-mouse IgG (Dakopatts) for 30 min at 4°C. After being washed with PBS containing 2% FBS, the cells were fixed with 5% formalin in PBS and analysed by using a Becton Dickinson FACScan system.

Northern blot hybridization. Total cellular RNA was extracted from cells grown to log phase as described previously (Chomczynski & Sacchi, 1987). Northern blot hybridization was carried out as described previously (Imai et al., 1991). The CD4 and β-actin probes were a 32P-labelled BamHI DNA fragment encoding the soluble extracellular region (368 amino acids) prepared from pAcYM1 containing the human CD4 gene (Morikawa et al., 1990) and a 32P-labelled β-actin gene, respectively.

Shedding of gp120. To test for shedding of gp120 by CD4 and related agents we used the method described by Moore et al. (1990). Briefly, HIV-1 virions were incubated at 37°C for 4 h in the presence of the test reagent. After incubation, the mixtures were applied to a Sepharose 4B column (2 ml) and fractions of 0.2 ml were collected. Each fraction was adjusted to 0.1% NP40 and incubated at 37°C for 30 min prior to ELISA to detect p24 and gp120.

Results

Kinetis of HIV-1 superinfection of L-2 cells

L-2 cells were isolated as a persistently HIV-1-infected clone from M10 cells after infection with the LAI strain (Barré-Sinoussi et al., 1983), and are copious producers of doughnut-shaped particles which are non-infectious and do not have RT activity (Goto et al., 1990; Yunoki et al., 1991). Only the precursor gag protein p53, but not the mature gag protein have been identified in L-2 cells by Western blot and immunoprecipitation analyses (Goto et al., 1990). Recently, epitope mapping using recombinant gag proteins has shown that MA b V17 recognizes the C-terminal 12 amino acids of gag p18 proteins, and reacts with p18 but not its precursor, p53 (Saitoh et al., 1992), suggesting the possible use of this MA b for the detection of superinfection of L-2 cells by HTLV-IIIB. Therefore, superinfection kinetics were examined by an IF assay using this MA b.

L-2 cells were superinfected with serial 10-fold dilutions of the conditioned medium of MOLT-4/HTLV-IIIB culture at multiplicities of about 3-2 to 3-2 x 10^-7 TCID50/ml. As a control, uninfected M10 cells were also primarily infected with the HTLV-IIIB strain under the same conditions. The cells were harvested every 4 days and subjected to an IF test with MA b V17. The kinetics of the appearance of cleaved gag protein in L-2 cells after superinfection were very slow (Fig. 1b) compared with the kinetics of HIV-1 replication in primary infected M10 cells (Fig. 1a). The percentage of p18 antigen-expressing cells reached almost 100% in M10 cells 4 days after infection at a multiplicity of 3.2, 8 days after infection at multiplicities of 3.2 x 10^-1 to 3.2 x 10^-4 TCID50/ml, and 12 days after infection at multiplicities of 3.2 x 10^-3 and 3.2 x 10^-6 TCID50/ml. By contrast, the percentage of antigen-expressing cells reached almost 100% in L-2 cells 12 days after superinfection at multiplicities of 3.2 and 3.2 x 10^-1 TCID50/ml, 16 days after superinfection at 3.2 x 10^-2 and 3.2 x 10^-3 TCID50/ml and 20 days after superinfection at 3.2 x 10^-4 TCID50/ml. No IF-positive cells were induced in L-2 cells 20 days after superinfection at multiplicities of less than 3.2 x 10^-5 TCID50/ml. Comparable kinetics were also observed for the appearance of RT activity in the culture fluid of superinfected L-2 cells (Fig. 2). This result indicates that the induction of protein reactive with MA b V17 in L-2 cells was positive evidence for superinfection of L-2 cells. Similarly, the production of infectious HIV-1 particles in L-2 cells after superinfection showed slow kinetics compared with those of primary HIV-1 infection in M10 cells (not shown).

Inhibition of the HIV-1 superinfection with CD4-related reagents

To clarify the possible involvement of the CD4 molecule in the superinfection of L-2 cells, we examined the inhibitory effects of several CD4-related reagents, i.e. synthetic CD4 peptides such as CD4(68-130), CD4(66-92) and CD4(45-60), sCD4, and anti-CD4 MAbs such as Leu-3a, OKT4A, OKT4 and #35. On primary infection of M10 cells with the HTLV-IIIB strain, CD4(68-130) and -(66-92) peptides, and sCD4 showed a significant decrease of HIV-1 infection at final concentrations of more than 125 500 and 0.5 µg/ml, respectively (Ohki
Fig. 1. Comparison of the kinetics of HIV-1 superinfection of L-2 cells and primary HIV-1 infection of M10 cells. M10 cells (a) and L-2 cells (b) were infected with HIV-1 at different multiplicities. Serial 10-fold dilutions (10 to 10^{-7}) of the conditioned medium (3.2 x 10^{7} TCID_{50}/ml) prepared from a MOLT-4/HTLV-IIIB cell culture were used as inocula. Cells (1 x 10^{6}) were mixed with 1 ml of the conditioned medium and incubated for 24 h at 37 °C. Cells were infected with (■) 3.2, (■) 3.2 x 10^{-1}, (▲) 3.2 x 10^{-2}, (●) 3.2 x 10^{-3}, (▼) 3.2 x 10^{-4}, (◊) 3.2 x 10^{-5}, (□) 3.2 x 10^{-6}, and (△) 3.2 x 10^{-7}/TCID_{50}/ml. After being washed with complete medium, the cells were cultured in complete medium. The cell number was counted every 4 days and adjusted to 5 x 10^{5} cells/ml in fresh complete medium. The cells harvested every 4 days were smeared, fixed with cold acetone, and then subjected to IF with MAb V17 to detect HIV-1 antigen expression.

Fig. 2. The kinetics of HIV-1 superinfection in L-2 cells as judged by RT assay. Culture fluids harvested every 4 days from the L-2 cell cultures superinfected at multiplicities of 3.2, 3.2 x 10^{-2}, and 3.2 x 10^{-4} TCID_{50}/ml were analysed for RT activity as described in Methods. Columns 1 to 5 represent samples taken 4, 8, 12, 16 and 20 days post-infection.

et al., 1992). A similar decrease of HIV-1 infection was obtained using MAbs Leu-3a and #35 at final concentrations of 5 and 200 μg/ml, respectively. Such a decrease of HIV-1 infection was not observed using OKT4A and OKT4 MABs at final concentrations of 5 μg/ml, although HIV-1-induced syncytium formation was strongly inhibited by MAB OKT4A even at a final concentration of 0.1 μg/ml (Ohki et al., 1992). Similar results have also been observed previously, i.e. Leu-3a and OKT4A are effective at inhibiting syncytium formation at 0.156 and 0.075 μg/ml, respectively, but not so effective at inhibiting HIV-1 infection, even at 2.5 μg/ml (Rey et al., 1991). These results suggest that a greater amount of MAB might be necessary to block HIV-1 infection than to block syncytium formation. Therefore, we examined the inhibitory effects of these CD4-related reagents on the superinfection of L2 cells at a variety of m.o.i.s and at concentrations similar to those used for the inhibition of primary HIV-1 infection.

The effect of these CD4-related reagents on the superinfection of L-2 cells at a multiplicity of 3-2 was first examined as described in Methods. As shown in Fig. 3(a), CD4(68–130) peptide completely blocked the superinfection at a final concentration of 1 mg/ml and this effect showed dose dependence. Slightly lower inhibitory effects were also obtained using sCD4 protein at a concentration of 1 μg/ml, and this protein also showed dose dependence. The inhibitory effect of CD4(66–92) peptide at a final concentration of 1 mg/ml was much lower when compared with that of CD4(68–130) peptide, but significantly delayed the superinfection kinetics. CD4(45–60) peptide showed kinetics very similar to those of the untreated control. Next, the inhibitory effects of these CD4-related reagents were examined with superinfections at lower multiplicity,
Fig. 3. The inhibitory effects of several CD4-related reagents on HIV-1 superinfection of L-2 cells at different multiplicities. L-2 cells were superinfected with the conditioned medium of MOLT-4/HTLV-IIIB cells at multiplicities of about 3·2×10^{-2} (a), 3·2×10^{-2} (b) and 3·2×10^{-4} (c). The inhibitory effects of several CD4-related reagents were examined as described in Methods. The CD4-related reagents used were as follows: CD4(68–130), CD4(66–92) and CD4(45–60) peptides at a final concentration of 1 mg/ml (○), 0·5 mg/ml (●), 0·25 mg/ml (▲) and 0·125 mg/ml (●); sCD4 at a final concentration of 1 μg/ml (●), 0·5 μg/ml ( ■), 0·25 μg/ml (▲) and 0·125 μg/ml (●); Leu-3a, OKT4A, and OKT4 MAbs at a final concentration of 5 μg/ml (●); and MAb #35 at a final concentration of 200 μg/ml (●). As controls, L-2 cells were mock-superinfected (○) or superinfected with HIV-1 (●). Furthermore, the L-2 cells were superinfected in the presence of AZT at a final concentration of 10 μg/ml and cultured in the presence of AZT at the same concentration. The appearance of HIV-1 antigen in the superinfected L-2 cells was determined by an IF test with MAb V17 as described in the legend to Fig. 1.

3·2×10^{-2} (Fig. 3b) and 3·2×10^{-4} TCID_{50}/ml (Fig. 3c). The inhibition exhibited by these CD4-related reagents was greater at lower m.o.i. Notably, almost complete blocking of the superinfection at a multiplicity of 3·2×10^{-4} TCID_{50}/ml was obtained using CD4(68–130) peptide at a final concentration of 125 μg/ml and sCD4 at a final concentration of 1 μg/ml (Fig. 3c). Similarly, the inhibitory effects of CD4(66–92) peptide at final concentrations of 1 mg/ml and 500 μg/ml were more apparent for the superinfections at multiplicities of 3·2×10^{-2} and
3.2 × 10⁻⁴ TCID₅₀/ml (Fig. 3b and c, respectively) than that of the peptide at a final concentration of 1 mg/ml or an m.o.i. of 3-2 (Fig. 3a). In contrast, CD4(45-60) peptide at a final concentration of 1 mg/ml was not inhibitory for superinfection even at a multiplicity of 3.2 × 10⁻⁴ TCID₅₀/ml (Fig. 3c). On the other hand, MAb Leu-3a at a final concentration of 5 µg/ml was less effective, although it did significantly delay the superinfection kinetics at a multiplicity of 3.2 × 10⁻⁴, whereas MAb Leu-3a at a final concentration of 10 µg/ml was significantly delayed the superinfection kinetics when a multiplicity of 3-2 x 10⁻⁴ TCID₅₀/ml was used (Fig. 3c). Thus, the involvement of the CD4 molecule in the superinfection of L-2 cells was clear, although there was a discrepancy in the inhibitory effects shown by CD4 peptides and anti-CD4 MAbs. Superinfection was completely inhibited by the CD4(68-
Fig. 5. Northern blot analysis of the CD4 transcript in L-2 cells. Total RNA fractions extracted from M10 (lanes 1 and 3) and L-2 (lanes 2 and 4) cells, which were electrophoresed and blotted onto a nylon membrane, were hybridized with CD4 probe (lanes 1 and 2). As an internal control, the same membrane was hybridized with β-actin probe (lanes 3 and 4) after removal of the previous CD4 probe.

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Fig. 5. Northern blot analysis of the CD4 transcript in L-2 cells. Total RNA fractions extracted from M10 (lanes 1 and 3) and L-2 (lanes 2 and 4) cells, which were electrophoresed and blotted onto a nylon membrane, were hybridized with CD4 probe (lanes 1 and 2). As an internal control, the same membrane was hybridized with β-actin probe (lanes 3 and 4) after removal of the previous CD4 probe.

130) peptide, but only partially by anti-CD4(68-130) MAb #35. Similarly, the superinfection was significantly inhibited by MAb Leu-3a, but not by the CD4(45-60) peptide. In addition, no inhibitory effect of MAb OKT4A on superinfection was observed, in contrast to its effect on primary HIV-1 infection (Ohki et al., 1992).

AZT treatment showed a strong blocking effect on the superinfection, indicating that RT was necessary for superinfection in L-2 cells.

Restricted CD4 down-modulation from surface of L-2 cells

The L-2 cell clone is permissive for HIV-1 superinfection, which appeared to be mediated by the cell surface CD4 molecule. On the other hand, another cell clone, MOLT-#8/IIIB-14 was found to be resistant to HIV-1 superinfection, even at a multiplicity of 3-2 TCID₅₀/ml (not shown). Several cell clones were isolated from MOLT-#8 after adsorption of the HTLV-IIIB strain at an m.o.i. of about 1 for 1 h at 37 °C by limiting dilution in a 96-well microplate. One of these clones, MOLT-#8/IIIB-14, did not produce RT-positive particles, although the cells expressed HIV-1 antigens. After superinfection with HTLV-IIIB at a multiplicity of 3-2 TCID₅₀/ml, the appearance of RT activity in the culture fluid was monitored under the same conditions as in the case of L-2 cells. However, no RT activity was detected during at least 42 days. Therefore we examined CD4 expression on the surface of L-2 and MOLT-#8/IIIB-14 cells by flow cytometry using several anti-CD4 MAbs (Fig. 4). M10 and MOLT-#8 cells, which were used as host cells for the preparation of L-2 and MOLT-#8/IIIB-14 respectively, were used as controls. The results of flow cytometry with MAbs Leu-3a and OKT4A in both L-2 and MOLT-#8/IIIB-14 cell clones were consistent with the results reported previously (Hoxie et al., 1986; Stevenson et al., 1987; Salmon et al., 1988), i.e. an almost complete depletion of their epitopes from the cell surface. Loss of the epitopes recognized by these MAbs from the surfaces of L-2 and MOLT-#8/IIIB-14 cells is probably due to the high expression of gp120 on the surface of these cells, as shown by flow cytometry with MAb 0.5β (Fig. 4). In contrast, it was found that the epitope region recognized by MAb OKT4 was still detectable on the surface of L-2, but not on the surface of MOLT-#8/IIIB-14 cells, although the relative IF intensity of the positive L-2 cells was only 6% of that observed with uninfected M10 cells. Thus, CD4 regions other than the primary gp120-binding site seem to remain exposed on the surface of L-2, but not on the surface of MOLT-#8/IIIB-14 cells. MAb #35 reacted only faintly with the surfaces of L-2 as well as M10 cells, whereas no significant reaction was observed between with MAb #35 and MOLT-#8/IIIB-14 or MOLT-8 cells.

Levels of CD4 mRNA and internal CD4 antigen are unaltered in L-2 cells

To identify the CD4 down-modulation mechanism in L-2 cells, we performed two experiments, one to identify the level of CD4 mRNA in L-2 cells and the other to identify intracellular CD4 protein by an IF test of acetone-fixed cells using several anti-CD4 MAbs. The level of expression of CD4 mRNA in L-2 cells was determined by Northern blot analysis. As an internal control, a β-actin probe was used. As shown in Fig. 5, the amount of CD4 transcript identified in L-2 cells was similar to that in uninfected M10 cells. Therefore, CD4 protein should be detectable in L-2 cells, although flow cytometry had shown severe CD4 down-modulation on the surface of L-2 cells (Fig. 4). In fact, the CD4 molecule was detected in L-2 cells at a level similar to that in M10 cells when MAbs OKT4 and #35 were used for the detection of intracellular CD4 protein by an IF test (Fig. 6). However, significant IF was not detected in L-2 cells using MAbs Leu-3a and OKT4A. These results suggest that CD4 molecules were synthesized in L-2 cells at almost normal levels, but that most of them remained inside the cell. The evidence of the reactivity of the CD4 molecules with MAbs OKT4 and #35, but not with anti-
Fig. 6. IF test of intracellular forms of CD4 molecule in M10 and L-2 cells. The M10 (a to f) and L-2 (g to l) cells were smeared, fixed with cold acetone, and then reacted with Leu-3a (a and g), OKT4A (b and h), OKT4 (c and i), #35 (d and j), 0.5β (e and k) MAbs, or normal mouse IgG (f and l).
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Fig. 7. Effect of CDR3-related reagents on gp120 shedding. The assay was done as described (Moore et al., 1990) using the ELISA to detect p24 (O) and gp120 (●). Before application to the Sepharose 4B column, HIV-1 particles in the superinfection inoculum were treated as described in Methods with RPMI 1640 medium supplemented with 5% FBS (a), CD4(68-130) at a final concentration of 0.125 mg/ml (b), CD4(66-92) at a final concentration of 0.5 mg/ml (c) or sCD4 at a final concentration of 1 μg/ml (d). The concentrations of CD4 peptide chosen were the same as those used for the inhibition of HIV-1 superinfection (Fig. 3c). Arrows indicate the elution volume of recombinant gp120 run in parallel experiments.

Leu-3a and OKT4A MAb's, indicated the formation of complexes with env MAb's, as shown previously in other HIV-1-infected cell systems (Kawamura et al., 1989; Buonocore & Rose, 1990; Crise et al., 1990; Koga et al., 1990).

Blocking of superinfection of L-2 cells by CDR3-related reagents is not due to gp120 shedding

Soluble CD4 has been shown to strip gp120 from the surface of some but not all HIV virions (Moore et al., 1990, 1992), and some peptides related to the CDR3 region of CD4 have been shown to mimic this effect (Berger et al., 1991). Although the peptides used by Berger et al. were highly derivatized and ours were not, it remained possible that inhibition of superinfection by our CDR3-related reagents was due to gp120 shedding. To control for this possibility, we assessed the ability of each competitive reagent to strip gp120 from HTLV-IIIB as described by Moore et al. (1990). At the concentrations used for the inhibition experiments described in Fig 3(c) we observed no shedding of gp120 from the superinfection inoculum during a 4 h incubation at 37°C with sCD4, CD4(68–130) or CD4(66–92) (Fig. 7). At higher concentrations of sCD4, shedding of gp120 was evident (data not shown) as reported by others (Moore et al., 1990; Berger et al., 1991). We conclude that superinfection inhibition in our experiments is the result of competition by CDR3-related reagents for the site of virus attachment to L-2 cells, and not to gp120 shedding from the superinfecting virus.

Discussion

The inhibition of superinfection shown by several CD4-related reagents clearly demonstrate the involvement of the CD4 molecule in HIV-1 superinfection of L-2 cells (Fig. 3). Therefore we characterized CD4 down-modulation on the surface of two cell clones expressing HIV-1 env proteins, i.e. superinfection-sensitive L-2 (Fig. 1 and 2) and resistant MOLT-#8/IIIB-14 (not shown) cells. Flow cytometry of L-2 cells revealed that the epitopes recognized by MAbs Leu-3a and OKT4A, near the CDR2-related region in the V1 domain (Landau et al., 1988; Mizukami et al., 1988; Peterson & Seed, 1988; Sattentau et al., 1989), were almost completely occluded from the surface of L-2 cells (Fig. 4). However, the epitopes recognized by MAbs OKT4 (V3 and V4) (Berger et al., 1988) and #35 (amino acid residues 72 to 84, which lie just before the CDR3-related region in the V1 domain) (Ohki et al., 1992) were still exposed on the cell surface of L-2 cells, although the relative amount of CD4 detected by MAb OKT4 was only 6% of that on
M10 cells (Fig. 4). The continual presence of the epitope recognized by MAb OKT4 on the cell surface has been observed previously even after complete loss of the epitope recognized by Mab Leu-3a during acute HIV-1 replication (Salmon et al., 1988). In contrast, flow cytometry of an HIV-1 superinfection-resistant cell clone, MOLT-#8/IIB-14, showed no exposure of these CD4 epitopes on the cell surface (Fig. 4). Therefore, the failure to superinfect MOLT-#8/IIB-14 cells with HIV-1 might be due to complete loss of the CD4 molecule from the cell surface.

The amount of the CD4 transcript in L-2 cells was similar to that in M10 cells (Fig. 5), consistent with previous results dealing with persistently HIV-1-infected cells (Stevenson et al., 1987), but not with those dealing with the acute phase of HIV-1-infected cells (Salmon et al., 1988). In fact, IF tests have revealed that MAbs OKT4 and #35 react with the intracellular CD4 molecule in acetone-fixed L-2 cells at a level similar to that in M10 cells (Fig. 6), even though the expression of CD4 on the surface of L-2 cells is greatly reduced compared with that on M10 cells (Fig. 4). On the other hand, most of the CDR2-related region seemed to be masked by HIV-1 env proteins as shown by IF tests with Leu-3a, OKT4A, and 0.5β MAbs, i.e. strong IF in M10 but only faint IF in L-2 cells using MAbs Leu-3a and OKT4A, but strong IF only in L-2 cells using MAb 0.5β. However, MAb 0.5β could react with HIV-1 env proteins complexed with the CD4 molecule because it recognizes the V3 region which is distinct from the CD4-binding site (Matsushita et al., 1988). Thus, it is indicated that at least the CDR3-related region, but not the CDR2-related region, is present as a freely accessible epitope inside L-2 cells at levels almost similar to those found in M10 cells. Furthermore, our data suggest that the CDR3-related region or the region close to it might act as a major receptor site for HIV-1 superinfection of L-2 cells for the following reasons. First, CD4(68-130) and CD4(66-92) peptides specifically blocked the superinfection as well as sCD4 protein (Fig. 3). Second, the epitopes recognized by MAbs #35 and OKT4 remain exposed on L-2 cells (Fig. 4). In addition, the epitope recognized by MAbs #35 is included in the CD4(66-92) peptide. Inhibition by these reagents was also shown not to be due to gp120 shedding. However, we cannot rule out the possibility that the CD4 CDR2-related region might play some part in the interaction with gp120 in the superinfection, because slight, but significant, delays in the superinfection kinetics were observed using MAb Leu-3a when a low multiplicity was used for superinfection (Fig. 3). The level of expression of the CDR2-related region of CD4 on the surface of L-2 cells might be so low that the detection of the epitope was impossible by flow cytometry (Fig. 4), although a low, but significant, level of IF was detectable inside L-2 cells (Fig. 6). If that is the case, then the amount of the CD4 molecule on the L-2 cell surface might play a role as a receptor for superinfection.

Interference between HIV-1 strains in our superinfection system using the persistently HIV-1-infected L-2 cell clone was incomplete; the L-2 cells could be superinfect- ed with HIV-1, although superinfection kinetics were very slow compared with primary infection of M10 cells (Fig. 1). The major reason for the incomplete interference was a small amount of CD4 remaining on the surface of L-2 cells which escaped down-modulation. These results might be essentially consistent with the reinfection events of already infected and infectious HIV-1-producing cells as suggested by the evidence that unintegrated HIV-1 DNA accumulates in several cases of chronically infected cells without superinfection with exogenously added HIV-1 (Pauza et al., 1990; Robinson & Zinkus, 1990; Besanski et al., 1991). In this case, CD4-dependent reinfection is observed even though the level of CD4 on the cell surface is undetectable by flow cytometry (Besanski et al., 1991). A large number of cells may be persistently or latently infected with HIV-1 in asymptomatic carriers. The superinfection mechanism described here could be a useful model for understanding the pathogenicity of AIDS.

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