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Mutations in the *env* gene of human immunodeficiency virus type 1 NDK isolates and the use of African green monkey CXCR4 as a co-receptor in COS-7 cells

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A previous report from this laboratory described the isolation of the first CD4-independent human immunodeficiency virus type 1 isolate, m7NDK. This independence of CD4 is due to seven mutations located in the C2, V3 and C3 regions of the gp120 protein. The present report describes the entry features of the m5NDK virus, which contains five of the seven m7NDK mutations, located in the V3 loop and C3 region. The entry of this virus is strictly CD4-dependent but it can fuse with African green monkey (agm) COS-7 cells bearing human CD4 (h-CD4). This fusion is directly due to the five mutations in the *env* gene. It has also been shown that entry of m7NDK is CD4-independent in COS-7 cells. Since the wild-type NDK and m7NDK viruses use the human CXCR4 protein as co-receptor, agm-CXCR4 was cloned and used in transfection and fusion inhibition experiments to show that this receptor can be used by the m5 and m7NDK viruses. The wild-type NDK virus, which does not enter COS-7 cells, can use agm-CXCR4, but only when the receptor is transfected into target cells. Although co-receptor nature and expression levels are still major determinants of virus entry, this is the first case where a few mutations in the *env* gene can overcome this restriction.

Introduction

CD4 is the primary receptor for the human immunodeficiency viruses HIV-1 and HIV-2, but binding to it is not sufficient for infection. Membrane fusion requires a cell-surface cofactor (or co-receptor), which belongs to the family of seven-transmembrane-domain G-coupled protein receptors (Berson *et al.*, 1996) (for reviews see Berson & Doms, 1998; Doms & Peiper, 1997;Moore *et al.*, 1997). A number of cofactors have been identified, the most common being CXCR4 (Feng *et al.*, 1996) and CCR5 (Alkhatib *et al.*, 1996; Deng *et al.*, 1996; Dragic *et al.*, 1996). CXCR4 and CCR5 allow membrane fusion with X4 and R5 strains, respectively (Berger *et al.*, 1998). Other receptors have been characterized *in vitro* and allow less efficient virus entry; they include CCR2b (Zhang *et al.*, 1997), CCR3 (He *et al.*, 1997; Rucker *et al.*, 1997), CCR8 (Rucker *et al.*, 1997), CX3CR1/V28 (Combadiere *et al.*, 1998; He *et al.*, 1997; Reeves *et al.*, 1997; Rucker *et al.*, 1997), US28 (Pleskoff *et al.*, 1997 *b*), STRL33 (or Bonzo) (Alkhatib *et al.*, 1997; Deng *et al.*, 1997; Li *et al.*, 1990; Liao *et al.*, 1997), GPR15 (or Bob) (Deng *et al.*, 1997; Farzan *et al.*, 1997), apj and CCR9 (Choe *et al.*, 1998).

A few HIV isolates can use simian (rhesus and fascicularis monkey) (Himathongkham & Luciw, 1996), murine (Bieniasz *et al.*, 1997; Tachibana *et al.*, 1997), feline (Willett *et al.*, 1997) or rat (Pleskoff *et al.*, 1997*a*) CXCR4 when the receptor is expressed in transfected human CD4-positive, CXCR4-negative cells. No HIV enters the simian cell line COS-7, derived from African green monkey (agm), under normal conditions of co-receptor expression. The nature of the co-receptor, the cell type producing the co-receptor and the presence of human (h-) CD4 seem to be the most important factors determining virus entry (Dimitrov, 1997).

While CD4 is almost always required, two CD4-independent HIV isolates have been characterized after long-term culture, the HIV-2 Rod/B isolate (Clapham *et al.*, 1992; Reeves & Schulz, 1997) and the HIV-1 m7NDK virus (Dumonceaux *et*

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al., 1998). This CD4-independent entry is due to mutations in the *env* gene, and both mutants use CXCR4 as receptor (Dumonceaux *et al.*, 1998; Endres *et al.*, 1996; Reeves *et al.*, 1997).

We have shown previously that a cluster of mutations in the C2, V3 and C3 regions of the m7NDK *env* gene are responsible for its ability to enter CD4-negative cells. We have proposed that these mutations in gp120 increase the affinity of the virus for CXCR4, thus allowing CD4-independent membrane fusion. This present paper describes the characterization of the m5NDK virus, which can fuse with simian COS-7 cells expressing h-CD4. This virus contains five of the seven mutations in m7NDK and these five mutations are responsible for its phenotype. The two other mutations in C2 in the *env* gene of m5NDK (which leads to m7NDK) result in a virus that can fuse with CD4-negative COS-7 cells. Expression of agm-CXCR4 by the non-permissive human U373MG cell line demonstrated that this protein is used as a co-receptor by m5 and m7NDK viruses. Fusion blockage experiments with the CXCR4-specific ligand SDF-1 supported this conclusion. We have also shown that the wild-type (wt) NDK virus, which cannot normally fuse with h-CD4-positive COS-7 cells, can do so only when agm-CXCR4 is overproduced in these cells. These results indicate, therefore, that the amount of expressed co-receptor is a major determinant of virus entry and that the five mutations in the *env* gene of m5NDK are sufficient to circumvent the very low virus fusion efficiency that results at the normal level of expression of the co-receptor.

Methods

■ **Cells and viruses.** Non-adherent cells were grown in RPMI 1640 medium (Gibco-BRL) supplemented with 5% foetal calf serum (FCS; Gibco-BRL), antibiotics and glutamine (Gibco-BRL). The CD4-positive human lymphoid T cell line CEM and NDK virus were gifts from F. Barré-Sinoussi (Institut Pasteur, Paris). All adherent cell lines were grown in DMEM (Eurobio) medium supplemented with 5% FCS, antibiotics and glutamine. HeLa–LTRLacZ, HeLa–CD4–LTRLacZ, COS-7–LTRLacZ and COS-7–CD4–LTRLacZ indicator cells have been described previously (Dragic & Alizon, 1993) and were gifts from M. Alizon (ICGM, Paris).

m5NDK and m7NDK viruses and the chronically infected CEM or HeLa cell lines have been described previously (Dumonceaux *et al.*, 1998).

■ **Plasmid constructs.** Plasmid pLTRLuc has been described previously (Dumonceaux *et al.*, 1998). The LTRLuc-PGK-hygro plasmid contains a luciferase gene under the control of the *Sac*I–*Hin*dIII long terminal repeat (LTR) fragment of HIV-1 NDK isolate and the hygromycin B resistance gene under the control of the phosphoglycerate kinase promoter (Adra *et al.*, 1987), both cloned into pBluescript SK(+) (Stratagene). The h-CXCR4 expression plasmid was a gift from B. Moser (University of Bern, Switzerland) (Loetscher *et al.*, 1994). The agm-CXCR4 sequence was amplified by RT–PCR and cloned into the pcDNA3 plasmid (Invitrogen) using the *Hin*dIII and *Xba*I sites. The pCMV-CD4 plasmid was constructed by inserting the CD4 coding sequence downstream of the CMV promoter using the *Eco*RI and *Bam*HI sites of the pUHD15-1 plasmid (Gossen & Bujard, 1992) and an *Eco*RI–*Pvu*I-digested SV40–puromycin resistance gene cassette was subcloned into the *Pvu*I and *Hin*dIII sites of pCMV-CD4 expression plasmid. pPGK-hygro^R was a gift from M. Alizon (ICGM, Paris). Replacing the hygromycin resistance gene with the CD4 cDNA resulted in pPGK-CD4.

■ **RT–PCR.** agm-CXCR4 was obtained after RT–PCR performed on COS-7 cell RNA. Total RNA was extracted from 5×10^6 COS-7 cells by using a commercial kit (Bioprobe, RNAB). Reverse transcription was performed on 10 µg total RNA by using the AMV RT kit (Promega). cDNAs were then purified on Centricon 30 (Amicon) and one-tenth of the resulting cDNA was used for amplification. PCR assays were performed in a Crocodile III cycler (Appligene) with the following amplification program: 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 53 °C for 30 s and 72 °C for 30 s, and 72 °C for 10 min. The primers used were 5′*Hin*dIII55 (5′ TTTAAATAAGCTTAGAACCAGCGGTTACCA 3′) and 3′*Xba*I1220 (5′ TTTAAATCTAGAAAGCAATAAAAACTGTACAATATTGGTC 3′). Five molecular clones were sequenced twice on both strands (see sequence analysis). The relevance of mutations was confirmed by PCR sequencing to determine consensus sequences.

■ **Cell clones.** U373 cells were co-transfected with pLTRLuc and the h-CXCR4 plasmid. Clones were selected with 500 µg/ml neomycin sulphate (Gibco-BRL). These cells were then co-transfected with the pPGK-CD4 and the pPGK-hygro^R plasmids to give the CD4–h-CXCR4–U373–LTRLuc cell lines. Clones were selected by using 0·15 mg/ml hygromycin B (Calbiochem).

U373 cells were transfected with LTRLuc-PGK-hygro plasmid to obtain U373–LTRLuc cells and clones were selected by using 0·15 mg/ml hygromycin B. These cells were then transfected with the agm-CXCR4 plasmid to yield agm-CXCR4–U373–LTRLuc cells, which were selected by using 0·5 mg/ml G418 (Gibco-BRL). The pCMV-CD4-SV-puro^R plasmid was then transfected into the agm-CXCR4–U373–LTRLuc cells to give CD4–agm-CXCR4–U373–LTRLuc cells, which were selected with 0·5 mg/ml G418, 0·3 mg/ml hygromycin B and 0·3 µg/ml puromycin (Calbiochem).

■ **Cell fusion assays.** Syncytium-formation assays were performed by using adherent or non-adherent cells infected with HIV-1 and adherent target cells. The indicator target cells contained transiently or stably transfected pLTRLuc reporter plasmid or stably transfected pLTRLacZ reporter plasmid. Cell ratios were usually 1 : 1 for adherent cell co-cultures and 1 : 2 to 1 : 5 for adherent/non-adherent cell co-cultures. Samples were analysed 8–16 h later, depending on cell fusion efficiency.

■ **Reporter assays.** Analyses were performed by using two indicator reporter gene systems (*lacZ* or luciferase), depending on the indicator cell used for *in situ* or quantitative analysis. *In situ* analysis measuring *β*-galactosidase activity used cells fixed in 0·5% glutaraldehyde and X-Gal assays were performed after incubation for 4 h at 37 °C or overnight at 4 °C (Dragic & Alizon, 1993). Blue-stained syncytia were scored under a binocular microscope. Quantitative analyses were made by using a LB9501 luminometer (EGG Wallace) (Sol *et al.*, 1993). Quantitative chloramphenicol red–*β*-galactopyranoside (CPRG) (Boehringer Mannheim) assays were also performed as described previously (Dumonceaux *et al.*, 1998).

■ **Blocking experiments.** SDF-1 (100–800 nM; R & D Systems) was added to 2×10^4 CD4-positive indicator cells per well in a 96-well plate 30 min before adding HeLa cells chronically infected with m7NDK (2×10^4 cells per well). Cells were co-cultured in triplicate and fusion efficiency was analysed 8 h later by a quantitative CPRG or luciferase test.

■ **Sequence analysis.** The *env* expression vector and the sequencing method used have been described previously (Dumonceaux *et al.*, 1998). The primers used to sequence the agm-CXCR4 plasmid were 5′*Hin*dIII55,

3′*Xba*I1220, 5′422 (5′ TGTCATCTACACAGTCAACCTCTACAGC 3′), 3′501 (5′ TTGGCCTCTGACTGTTGGTGGCGTG 3′), 5′540 (5′ GGCCAAGGAAGCTGTTGGCTG 3′) and pcDNA3-specific primers 5′pcDNA3 (5′ AAATTAATACGACTCACTATAGGGAGACCC 3′) and 3′pcDNA3 (5′ CAACTAGAAGGCACAGTCGAGCC 3′).

Results

The m5NDK entry phenotype

We have previously isolated the m5NDK virus, which contains five mutations in the C3 and V3 regions of the gp120 protein and whose entry into cells is strictly CD4-dependent (Fig. 1). We have now analysed the entry requirements of m5NDK by co-culturing CEM cells infected with wtNDK (Fig. 2*a*, *b*) or m5NDK (Fig. 2*c*, *d*) viruses with HeLa (Fig. 2*a*, *c*) or COS-7 (Fig. 2*b*, *d*) CD4-positive LTRLacZ indicator cells. CEM cells chronically infected with m7NDK virus were co-cultured with HeLa (Fig. 2*e*, *f*) or COS-7 (Fig. 2*g*, *h*) CD4-positive (Fig. 2*e*, *g*) or -negative (Fig. 2*f*, *h*) LTRLacZ indicator cells.

All CEM cells infected with either wt, m5 or m7NDK viruses formed syncytia with human CD4-positive cells (Fig. 2 *a*, *c*, *e*). Co-cultures with human CD4-negative cells and wt or m5NDK-infected cells formed no syncytia. In contrast, m7NDK-infected cells allowed fusion with CD4-negative human cells (Fig. 2*f*). Co-culture between cells infected with m5NDK and CD4-positive COS-7 cells also led to efficient syncytium formation (Fig. 2*d*) and this co-culture was 25–50-fold more efficient at forming syncytia than that with wtNDK-infected cells (Fig. 2*b*). CEM cells infected with m7NDK formed syncytia with both CD4-positive and -negative COS-7 cells (Fig. 2*g*, *h*). Similar results were obtained by using supernatants of wt, m5 and m7NDK viruses. These results demonstrate that the m5NDK virus enters human and simian CD4-positive cells, whereas the wtNDK virus only enters CD4-positive human cells, as expected. The CD4-independent m7NDK isolate fused with human and simian CD4-positive and -negative cells.

Effect of mutations in the C2, V3 and C3 regions

The mutations previously described in the C2, V3 and C3 regions of *env* were used as a basis for the correlation of tropism in simian cells with genetic changes, using chimeric constructs of m5 and wt *env* genes. A chimera corresponding to the mutations in the V3 and C3 regions of the m5NDK *env* gene inserted into the wt *env* gene was expressed in HeLa cells. These cells were cultured with indicator cells and fused with CD4-positive HeLa cells or CD4-positive COS-7 cells (Fig. 3). In contrast, the reverse chimera allowed only fusion with CD4-positive HeLa cells. CD4-negative indicator cells did not form syncytia. We conclude that the C3 and V3 mutations in the m5NDK gp120 protein are responsible for and sufficient to allow entry of m5NDK into CD4-positive COS-7 cells.

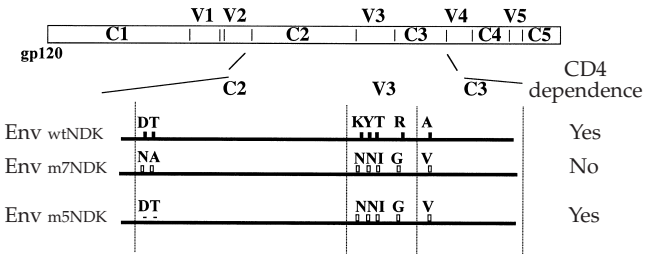

Fig. 1. Consensus sequence of *env* genes. Env wtNDK, sequence obtained from the wild-type NDK virus; Env m7NDK, sequence obtained from the CD4-independent mNDK virus; Env m5NDK, sequence obtained from the CD4-dependent mNDK virus.

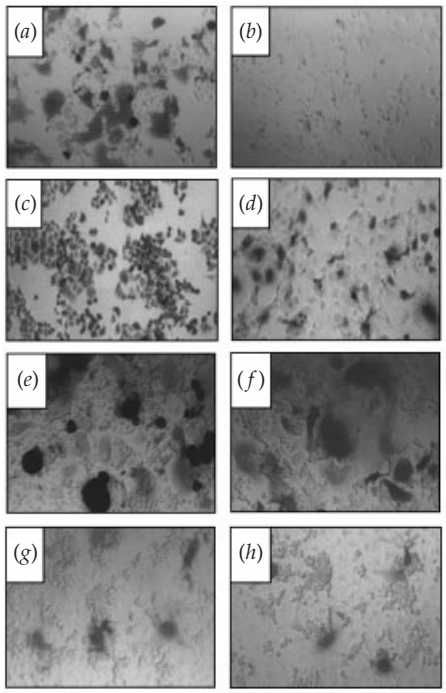

Fig. 2. Co-culture between CEM cells chronically infected with wt, m5 or m7NDK viruses and LTRLacZ target cells. (*a*)–(*d*) CD4-positive HeLa cells (*a*, *c*) or COS-7 cells (*b*, *d*) were cultured with CEM cells chronically infected with wtNDK (*a*, *b*) or m5NDK (*c*, *d*). (*e*)–(*h*) CEM cells chronically infected with m7NDK were cultured with HeLa (*e*, *f*) or COS-7 (*g*, *h*) CD4-positive (*e*, *g*) or CD4-negative (*f*, *h*) cells for 16 h. An *in situ* *β*-galactosidase test was performed to score and analyse specific fusion. Magnification, × 60.

The m7NDK virus enters CD4-negative agm cells. We had demonstrated previously that the two mutations in the C2 region are not sufficient to allow CD4-independent virus entry, but they ensured a CD4-independent entry phenotype in human cells when associated with the five mutations observed in the C3 and V3 regions (Dumonceaux *et al.*, 1998). We confirmed that the mutations in the C2 region played the same role in allowing CD4-independent simian cell entry by constructing a chimera corresponding to the m7NDK C2 region cloned in the m5NDK *env* gene context (Fig. 3). This

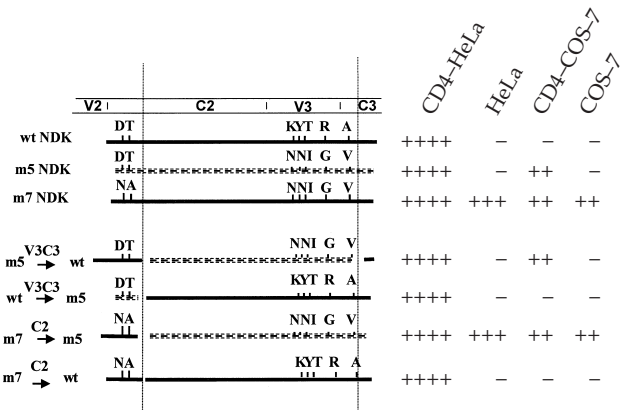

Fig. 3. Fusion phenotypes of chimeric *env* genes. Regions were exchanged by using *Eco*RV and *Hin*dIII sites for V3 and C3 regions and *Eco*RI and *Eco*RV sites for the C2 region. The fusion capacities of the resulting chimeric *env* expression vectors were analysed by culturing transiently transfected HeLa cells with CD4-positive or CD4-negative HeLa or COS-7 LacZ indicator cells for 16 h. β-galactosidase activity was assayed *in situ*. Results represent means of at least three to five independent experiments. Syncytium formation was scored as follows: –, < 5; ++, 50–200; +++, 200–1000; ++++, > 1000.

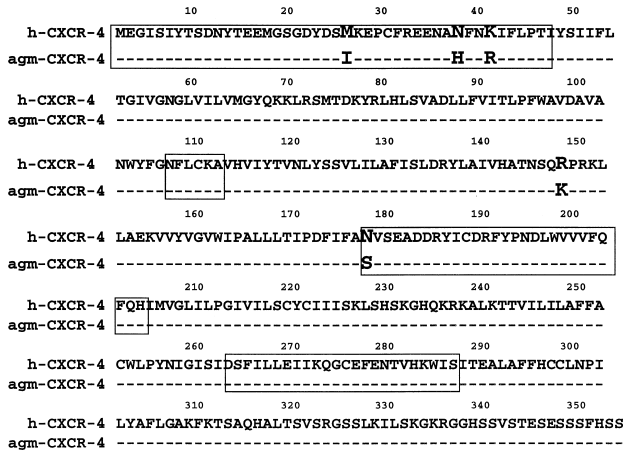

Fig. 4. Alignment of the sequences of the receptors encoded by h-CXCR4 and agm-CXCR4 cDNAs. Identical amino acids are indicated by dashes. Extracellular domains are boxed.

chimeric *env* gene allowed fusion between the transfected cells and CD4-positive or -negative HeLa or COS-7 LTRLacZ indicator cells (Fig. 3). Hence, the two mutations in the C2 region, in association with the five mutations in the C3 and V3 regions, are responsible for the CD4-independent entry of the virus into COS-7 cells. The two mutations in the C2 region alone are not sufficient to ensure entry into simian cells (Fig. 3).

Cloning of agm-CXCR4

As h-CXCR4 is the m7NDK isolate receptor, we postulated that agm-CXCR4 could act as the receptor for m5 and m7NDK virus. We therefore performed RT–PCR on COS-7 cell RNA

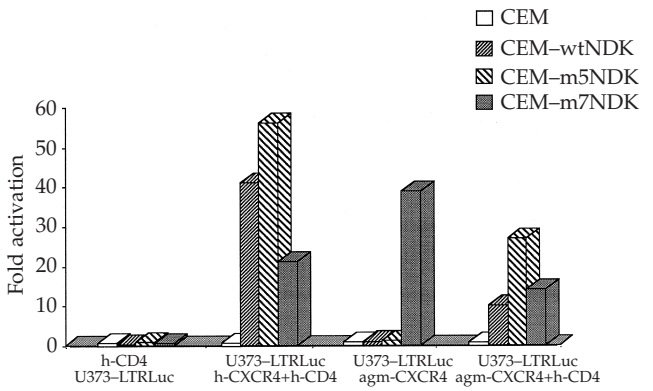

Fig. 5. agm-CXCR4 is used as a receptor by wtNDK, m5NDK and m7NDK isolates. h-CXCR4 or agm-CXCR4 were stably co-transfected with the HIV-1 LTR–luciferase vector into human CD4-positive or CD4-negative U373-MG cells. These transfected cells were then cultured with uninfected CEM cells or cells infected with either wt, m5 or m7NDK. Induction was standardized with reference to the luciferase activities obtained from cultures with the uninfected CEM cells. The experimental results shown represent those of three independent manipulations.

with oligonucleotides based on the h-CXCR4 sequence to isolate the agm-CXCR4 cDNA. This clearly demonstrated that agm-CXCR4 is expressed in COS-7 cells. We next subcloned the cDNA into a eukaryotic expression vector and compared its sequence with that of h-CXCR4. We found that five amino acids were different (Fig. 4); three in the N-terminal extracellular region ($M^{24} \rightarrow I^{24}$; $N^{35} \rightarrow H^{35}$; $K^{38} \rightarrow R^{38}$), one in the second intracellular loop ($R^{146} \rightarrow K^{146}$) and one in the second extracellular loop ($N^{176} \rightarrow S^{176}$). This last change corresponds to a potential *N*-glycosylation site in the h-CXCR4 protein.

Use of agm-CXCR4 by mNDK virus to enter COS-7 cells

The h- or agm-CXCR4 genes were transfected into U373–LTRLuc and CD4–U373–LTRLuc indicator cells. These cells were cultured with CEM cells chronically infected with either wt, m5 or m7NDK viruses. The reporter gene was not *trans*-activated when infected cells were cultured with CD4-positive U373–LTRLuc cells (Fig. 5), which confirmed that U373 cells are not infectable and that CD4 alone cannot be the sole receptor for HIV, as expected. There was a 40-fold *trans*-activation of the reporter gene when agm-CXCR4–U373 cells were cultured with cells chronically infected with m7NDK. No *trans*-activation occurred when they were cultured with the other infected cells, which is in agreement with the CD4-dependence of the related viruses. In contrast, there was an unexpected 10-fold *trans*-activation of luciferase expression after culturing agm-CXCR4–CD4–U373–LTRLuc cells with cells chronically infected with the wtNDK isolate (Fig. 5), and 27- and 14-fold activation, respectively, when agm-CXCR4–CD4–U373–LTRLuc cells were cultured with cells chronically infected with m5 or m7NDK viruses (Fig. 5). The co-cultures

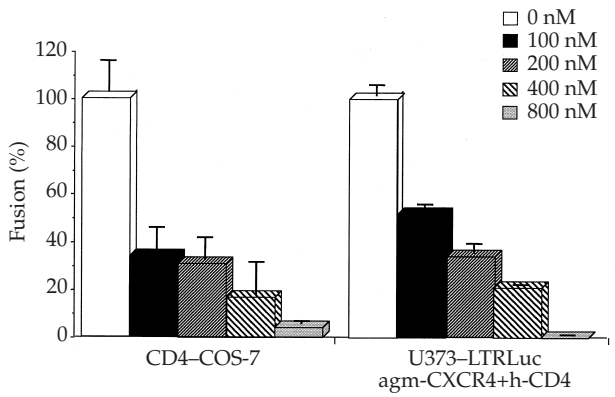

Fig. 6. Cell–cell fusion inhibition by SDF-1; fusion blocking experiment on CD4-positive COS-7 and agm-CXCR4–CD4–U373 cells. SDF-1 (100–800 nM) was added to CD4-positive COS-7 or CD4-positive agm-CXCR4–U373 indicator cells 30 min before adding HeLa cells chronically infected with m7NDK. Fusion efficiency was analysed 8 h later by quantitative CPRG–β-galactosidase (CD4–COS-7 cells) or luciferase tests (CD4–agm-CXCR4–U373 cells).

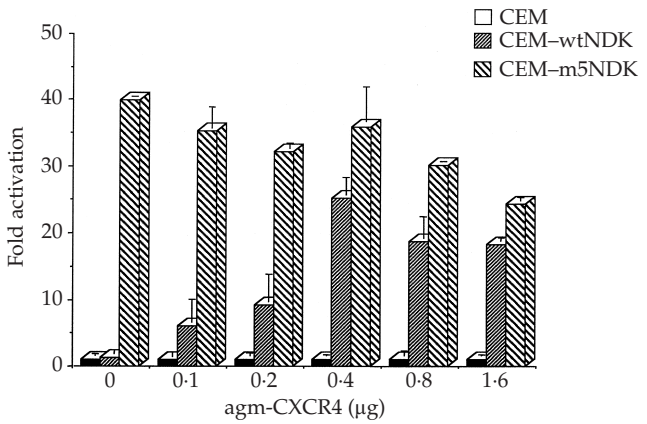

Fig. 7. Overproduction of agm-CXCR4 in CD4–COS-7 cells allows fusion with CEM cells infected with wtNDK. CD4-positive COS-7 cells were transiently co-transfected with different amounts of agm-CXCR4 (0, 0·1, 0·2, 0·4, 0·8 and 1·6 μg) with the pLTRLuc plasmid and co-cultured with CEM cells chronically infected with wt or m5NDK. Induction was standardized with reference to the luciferase activities obtained from cultures with uninfected CEM cells. Fold activation resulting from co-culture with CEM cells chronically infected with wtNDK or m5NDK isolates, respectively, was as follows: for 0 μg agm-CXCR4, 1·3 and 39·8; 0·1 μg, 6 and 35·7; 0·2 μg, 9·1 and 32·1; 0·4 μg, 25·2 and 35·8; 0·8 μg, 18·7 and 30; 1·6 μg, 18·2 and 24·4.

with CEM cells infected with the m7NDK virus were so efficient that most of the syncytia that were formed had died before the luciferase test was performed, leading to an erroneously low activity. Hence, agm-CXCR4 is sufficient for the HIV-1 isolates we tested to enter target cells.

SDF-1 blocking experiments

The natural ligand of h-CXCR4, h-SDF-1, blocks the interaction between CXCR4 and gp120. h-SDF-1 was added (100–800 nM) to CD4-positive COS-7–LTRLacZ or CD4-positive agm-CXCR4–U373–LTRLuc cells. Fusion between these cells and HeLa cells chronically infected with m7NDK virus was analysed 8 h later by a CPRG β-galactosidase test for COS-7 and by a luciferase test for U373 cells (Fig. 6). The effects of SDF-1 on CD4-dependent fusion were correlated with the concentration of SDF-1 used. At 100 nM, SDF-1 specifically inhibited syncytium formation in COS-7–LTRLacZ cells (34%) and agm-CXCR4–U373–LTRLuc cells (55%). An 8-fold increase in h-SDF-1 concentration blocked m7NDK fusion of CD4-positive agm-CXCR4–U373 cells totally (100%). This SDF-1 concentration also almost completely blocked fusion between CD4-positive LTRLacZ COS-7 and HeLa cells infected with m7NDK virus (96%) (Fig. 6).

We have also performed this fusion inhibition experiment on CD4-negative COS-7–LTRLacZ and agm-CXCR4–U373–LTRLuc cells and observed that the effects of h-SDF-1 were similar (data not shown).

While we cannot exclude the possibility that another receptor(s) is used in COS-7 cells, no other known co-receptor is expressed in U373 cells. Therefore, chemokine blocking experiments strongly suggest that agm-CXCR4 is the main receptor used by m5 and m7NDK to enter COS-7 cells.

The amount of agm-CXCR4 and virus entry

We postulated that the wtNDK isolate fuses with U373 cells bearing h-CD4 and agm-CXCR4 but not with h-CD4–COS-7 cells (which constitutively express agm-CXCR4), because of the relative agm-CXCR4 expression levels.

As no anti-agm-CXCR4 antibody was available and because anti-h-CXCR4 antibody did not recognize agm-CXCR4, we performed transient experiments to overexpress agm-CXCR4. If the amount of agm-CXCR4 indeed restricts wtNDK virus entry, the overexpression of this receptor in CD4-positive COS-7–LTRLuc cells should allow fusion with CEM cells chronically infected with these viruses. We thus transiently co-transfected CD4-positive COS-7 cells with pLTRLuc and different amounts of agm-CXCR4-expressing plasmid and co-cultured them with CEM cells infected with either wtNDK and m5NDK viruses (Fig. 7). As expected, no *trans*-activation was observed when the reporter gene alone was transfected into CD4-positive COS-7 cells after co-culture with CEM cells infected with wtNDK. When indicator cell lines were co-cultured with CEM cells chronically infected by m5NDK virus, we observed a 39·8-fold *trans*-activation of the luciferase gene. We did not observe a significant change in the degree of *trans*-activation when the amount of agm-CXCR4 was increased. However, when the co-culture was done with CEM cells chronically infected with wtNDK, we observed that the luciferase *trans*-activation level was directly correlated with the amount of agm-CXCR4 plasmid transfected, until a maximal level was attained (0·4 μg CXCR4) (Fig. 7). These results clearly demonstrate that CEM cells chronically infected with m5NDK

virus fuse efficiently with CD4-positive COS-7 cells independently of agm-CXCR4 expression, whereas fusion mediated by wtNDK virus needs to pass a threshold level of agm-CXCR4 expression to become efficient. The amount of agm-CXCR4 surface expression is thus an essential parameter that restricts wtNDK virus entry.

Discussion

We reported previously that the five mutations in the V3 loop ($K^{296}Y^{297}T^{298} \rightarrow N^{296}N^{297}I^{298}$, $R^{307} \rightarrow G^{307}$) and the C3 region ($A^{333} \rightarrow V^{333}$) (Fig. 1) of the *env* gene of m5NDK were not related to CD4-independent virus entry (Dumonceaux *et al*., 1998). The present report shows that CEM cells chronically infected with m5NDK virus fuse with CD4-positive COS-7 cells 25–50-fold more efficiently than do CEM cells chronically infected with wtNDK (Fig. 2).

We also demonstrated the importance of the C2 mutations for the CD4-independent entry of virus into COS-7 cells (Fig. 3). Indeed, we show here that the m7NDK virus can enter COS-7 cells in a CD4-independent manner. This result was expected, as we had previously found that m7NDK can enter cells independently of CD4 (Fig. 1). Analysis of *env* genes from cells infected with either wt, m5 or m7NDK strongly suggests that the viruses have undergone a gradual genetic drift. Since the m5NDK virus has five of the seven mutations observed in the m7NDK virus, the appearance of these mutations seems to correlate with an extension of the entry spectrum.

If m5 and m7NDK viruses use h-CXCR4 to enter human cells, they could use a simian equivalent to enter COS-7 cells. We checked this by cloning agm-CXCR4 after RT–PCR performed on COS-7 cell RNA (Fig. 4). We showed that the expression of this receptor was sufficient for m7NDK virus to enter non-permissive U373-MG cells (Fig. 5). Experiments with SDF-1 showed that only a high concentration of the chemokine (800 nM) blocked the fusion between HeLa cells infected with m7NDK viruses and CD4-negative (data not shown) or -positive COS-7 cells (Fig. 6). Nevertheless, as the results were similar for CD4-negative (data not shown) and -positive agm-CXCR4–U373 cells (where no other receptor is known to be expressed), these results strongly suggest that agm-CXCR4 is the main receptor used by the mNDK viruses to enter COS-7 cells.

In the same way, CEM cells infected with wtNDK virus can fuse with h-CD4–agm-CXCR4–U373 cells but not with h-CD4–COS-7 cells (Fig. 5). We postulated that there was more CXCR4 expressed in agm-CXCR4–U373 cells than in COS-7 cells. This overproduction could not be demonstrated by FACS analysis, as all the antibodies we tested (12G5, Mab171, Mab172 and Mab173; R & D Systems) did not recognize agm-CXCR4 (data not shown) and because no other specific antibody is available at present. The importance of CXCR4 overexpression was thus confirmed by transient transfection of agm-CXCR4 plasmid in CD4–COS-7 cells. The use of 0·4 μg agm-CXCR4 was sufficient to allow formation of syncytia with CEM cells infected with wtNDK virus, while untransfected cells did not form syncytia (Fig. 7). When we increased the amount of agm-CXCR4 transfected, there was no further significant change in the efficiency of fusion observed. These results indicate that CEM cells infected with wtNDK virus cannot fuse with COS-7 cells when there is a normal expression level of agm-CXCR4, whereas CEM cells infected with m5NDK isolate fuse efficiently under the same conditions. The m5NDK virus enters COS-7 cells once a threshold level of agm-CXCR4 expression is reached, whereas wtNDK entry into COS-7 cells requires an increase in agm-CXCR4 expression level to be efficient. These results complement the findings of Platt *et al*. (1998), who showed that infection of cultured cells by R5-tropic HIV-1 strains is extremely sensitive to the level of CD4 expression that allows efficient interactions with virus gp120s. Furthermore, previous studies have demonstrated that infection of HeLa-CD4 clones by T cell-adapted NL4-3 is independent of CD4 content only once a threshold expression level is reached (Kozak *et al*., 1997). Moreover, results obtained for CCR3 (Rucker *et al*., 1997) have shown that this receptor serves as a functional co-receptor for a wider spectrum of viruses when it is present at a high concentration. Taken together, these results indicate that the entry of HIV-1 depends greatly on the affinity of the envelope protein for its receptors (CD4, CXCR4) but also on the level of co-receptor expression. Nevertheless, the overproduction of a co-receptor does not allow entry of all HIV isolates; wtNDK virus cannot infect CD4-positive cells that transiently express rat CXCR4 (Pleskoff *et al*., 1997*a*).

Our results support the notion that, although the concentration of co-receptor is a major determinant of virus entry (Moore, 1997), some mutations in the *env* gene can overcome this restriction. The m5NDK isolate is thus the first example of a virus isolate in which a few mutations in the *env* gene can circumvent this hindrance to virus entry.

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