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Coreceptor Phenotype of Natural Human Immunodeficiency Virus with Nef Deleted Evolves In Vivo, Leading to Increased Virulence

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The Sydney Blood Bank Cohort is a group of patients with slowly progressive infection by a human immunodeficiency virus strain containing spontaneous deletions within the nef long terminal repeat region. In 1999, 18 years after the initial infection, one of the members (D36) developed AIDS. In this work, we used an ex vivo human lymphoid cell culture system to analyze two viral isolates obtained from this patient, one prior to the onset of AIDS in 1995 and one after disease progression in 1999. Both D36 isolates were less potent in depleting CD4+ T cells than a reference dualtropic, nef-bearing viral isolate. However, the 1999 isolate was measurably more cytotoxic to CD4+ T cells than the 1995 isolate. Interestingly, although both isolates were nearly equally potent in depleting CCR5+ CD4+ T cells, the cytotoxic effect of the 1999 isolate toward CCR5+ CD4+ T cells was significantly higher. Furthermore, GHOST cell infection assays and blocking experiments with the CXCR4 inhibitor AMD3100 showed that the later D36 1999 isolate could infect both CCR5+ and CCR5− CXCR4+ cells efficiently, while infection by the 1995 isolate was nearly completely restricted to CCR5+ cells. Sequence analysis of the V1/V2 and V3 regions of the viral envelope protein gp120 revealed that the more efficient CXCR4 usage of the later isolate might be caused by an additional potential N-glycosylation site in the V1/V2 loop. In conclusion, these data show that an in vivo evolution of the tropism of this nef-deleted strain toward an X4 phenotype was associated with a higher cytopathic potential and progression to AIDS.

In human immunodeficiency virus (HIV)-infected individuals, both viral and host factors regulate viral replication, depletion of CD4+ T cells, and disease progression. Host factors include genetic determinants such as the expression of coreceptors (17, 24, 60) and the ability to establish an efficient immune response (reviewed in references 41 and 50). Furthermore, mutations in the HIV envelope protein Env that cause a change in coreceptor usage have been shown to influence disease progression (14, 37, 55, 56, 65).

In many but not all patients, disease progression coincides with broadened coreceptor usage. HIV strains isolated early after infection encode Env proteins that utilize CCR5 as a coreceptor to enter host cells (R5 viruses), whereas viruses isolated at later disease stages often represent Env variants that can utilize CXCR4 (X4 viruses) or both CCR5 and CXCR4 (R5X4 viruses) (reviewed in reference 5). In addition, viral accessory genes such as rev, tat, vif, vpr, and vpu (42, 66, 68) and nef (16, 40) have been implicated in disease development. In particular, Nef is a multifunctional protein (46) that enhances viral replication (43, 59) and infectivity (13), modulates apoptosis (26, 49, 67), and decreases the expression of CD4 (1), the major histocompatibility (MHC) class I (57), and CD28 (61). Long-term studies have shown that disease progression is markedly delayed in humans or rhesus macaques infected with HIV or simian immunodeficiency virus (SIV) strains, respectively, that carry deletions in the nef gene (15, 16, 36). Interestingly, spontaneous partial repair of the nef gene in vivo has been correlated with enhanced infectivity and disease progression (10, 52).

One of the best-examined patient groups of long-term nonprogressors and long-term survivors is the Sydney Blood Bank Cohort (SBBC) (16), which consists of one blood donor (D36) and eight transfusion recipients who were infected with an HIV-1 strain containing multiple spontaneous alterations in the viral genome. These alterations include deletions in the nef open reading frame and a part of the long terminal repeat (LTR) region as well as duplications and rearrangements within the LTR. Three of the cohort members remained asymptomatic for at least 14 years and are classified as long-term nonprogressors (8), two died of causes unrelated to HIV, and one member with systemic lupus erythematosus died of causes possibly related to HIV (38). Three members had declining CD4 counts and detectable viral loads and therefore are grouped as long-term survivors (8). One of these long-term survivors, the original blood donor D36, developed AIDS 18 years after infection with this nef-deleted HIV-1 strain and started highly active antiretroviral therapy in 1999 (38). Sequence analysis of virus recovered from D36 in 1999 revealed additional deletions in the nef LTR region, thereby reducing the possibility that restored Nef caused the onset of AIDS (8).

In this study, we sought to determine whether the clinical
progression of patient D36 corresponds to changes in the virulence of HIV isolates from this patient and to identify possible mechanisms underlying such changes. With a modified version of ex vivo human lymphoid histoculture, we compared virus isolated from patient D36 prior to disease progression in 1995 (D36/95) and after progression but before therapy was initiated in 1999 (D36/99). We found that the later viral isolate was more cytotoxic than the earlier isolate and that this increased cytotoxicity was caused by more efficient CXCR4 usage and expanded target cell range.

MATERIALS AND METHODS

Preparation of viral stocks. NL4-3 was a gift from Malcolm Martin via the AIDS Research and Reference Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health. The molecular clone 49-5 was a gift from Bruce Chesebro. Infectious virus stocks were prepared by transfecting 293T cells with proviral DNA as described previously (2). The primary isolates D36/95, D36/99, 7/86, and 1/85 were expanded by infection of heterologous peripheral blood mononuclear cells (PBMC). Isolates 7/86 and 1/85 were gifts from Ruth Connor (14). The p24^ gag ^ concentrations of viral stocks were assessed by enzyme-linked immunosorbent assay (ELISA) (NEN Life Sciences, Boston, Mass.).

Culture and infection of human lymphoid tissues ex vivo. Human noninflammatory tonsil tissue removed during tonsillectomy (provided by the National Disease Research Interchange, Philadelphia, Pa., and the Kaiser hospitals in San Francisco, South San Francisco, and San Rafael) was prepared for lymphoid aggregate culture as previously described (23, 37). In brief, tonsil tissue was mechanically dispersed, and isolated cells were transferred to 96-well U-bottom plates at a concentration of 10^7 cells per ml, 200 μl per well. Cells were allowed to aggregate at the bottom of the well and were not dispersed for the remainder of the culture period. Ex vivo human lymphoid cell cultures were inoculated within 24 h of preparation with HIV-1 at 80% 50% tissue culture infective doses, as determined by the terminal dilution of the virus stocks in quasiduplicate on heterologous phothemagglutin-activated PBMC as described previously (45).

Assessment of CD4^+ T-cell depletion by FACS analysis. At the indicated time points, cells from infected and uninfected lymphoid cell cultures were stained for cell surface markers CD3, CD4, CD8, and CCR5 as described previously (45, 55) with the following monoclonal antibodies: anti-CD3 (clone SK7, phycoerythrin [PE] conjugated), anti-CD4 (clone SK3, fluorescein isothiocyanate [FITC] conjugated), anti-CD8 (clone SK1, peridinin chlorophyll protein [PerCP] conjugated), anti-CD4 (clone SK3, fluorescein isothiocyanate [FITC] conjugated) (Becton Dickinson), and anti-CCR5 (clone 2D7, allophycocyanin conjugated) (PharMingen). Then, 10,000 lymphocytes positive for CD3 surface marker were counted by fluorescence-activated cell sorting (FACS), and the data were analyzed by Cellquest software (Becton Dickinson). To facilitate comparison among experiments, CD4^+ T-cell depletion was assessed by measuring the ratio of CD4^+ to CD8^+ T cells. This value was normalized to the CD4/CD8 ratio of control (uninfected) samples.

Measurement of apoptosis. At the indicated time points, cells from infected and uninfected lymphoid cell cultures were washed with phosphate-buffered saline—2% fetal bovine serum—2.5 mM CaCl_2, stained for cell surface markers CD3, CD4, and either annexin V-phycocerythrin (Alexis) or 200 nM tetramethylrhodamine methyl ester (TMRM; Molecular Probes) for 30 min at room temperature, washed again, and subjected to flow cytometric analysis. To determine activation of caspase-3, cells were incubated with 10 μM PhiPhiLux-G1D2 (Alexis) for 1 h at 37°C, stained, washed for CD3 and CD4, and subjected to FACS analysis. GHOST cell and HeLa infection assays. GHOST cell assays were performed as reported previously (11). Briefly, 20,000 CXCR4^+ CD^4^+ (GHOST-X4) or CCR5^+ CD^4^+ GHOST (GHOST-R5) cells were plated in 12-well plates and infected at a multiplicity of infection (MOI) of 0.1. At 72 h after infection, infected cells were identified by flow cytometric analysis. CXCR4 or CCR5^+ HeLa CD4 cells were pretreated for at least 12 h with the indicated concentrations of AMD3100 and inoculated with HIV at an MOI of 0.01. At 72 h after infection, the concentration of p24^ gag ^ in the supernatant was assessed by anti-p24 ELISA.

Sequence analysis of the gp120 V1/V2 and V3 regions of D36/99. CXCR4^+ CCR5^+ CD^4^+ HeLa cells were infected with D36/99 at an MOI of 0.5. At 12 days after infection, genomic DNA was isolated with the Qiagen DNeasy Tissue kit. The gp120 gene was PCR amplified with the following primers at an annealing temperature of 51°C: 5′ primer D36-7089rev, TGGTGTCACAGTCTATTAT G6; 3′ primer D36-7089rev, TGGTGTCACCTCCTAATGGT. Specific bands were purified from an agarose gel with the Qiagen gel extraction kit. PCR products were cloned into the pcCR2.1 vector (Invitrogen). Inserts of two clones were sequenced with the M13 reverse sequencing primer (CAGGAAACAGCT ATGAC) and the M13 sequencing (−20) primer (GTAAAACGACGGCCAG T).

RESULTS

Characterization of the virulence of D36/95 and D36/99. First, the clinical isolates from patient D36 were tested for their potential to deplete CD4^+ T cells in ex vivo human lymphoid cultures. The kinetics of HIV-induced CD4^+ T-lymphocyte depletion was measured by staining cultures with antibodies to CD3, CD4, and CD8 and displayed as a ratio of CD4^+ to CD8^+ T cells as described previously (23, 37, 45, 54, 55). We compared the two D36 isolates with two other primary isolates from a different cohort. These isolates, called 7/86, an R5X4 strain, and 1/85, an R5 strain (14), are wild-type nef strains. As expected, the number of CD4^+ T cells in 1/85-infected cultures decreased only slightly over time, since R5 strains can infect and deplete only the small subset of CCR5-expressing CD4^+ T cells (28, 45, 54, 55). In contrast, the dualtropic virus 7/86 depleted CD4^+ T cells markedly due to its expanded target cell range (Fig. 1A) (55).

Overall, CD4 depletion was less severe in cultures infected with either of the two D36 isolates than in cultures infected with the R5X4 reference strain 7/86 (Fig. 1A). However, the later D36/99 isolate was measurably more cytopathic than the earlier D36/95 isolate, with more severe CD4^+ T-cell depletion despite comparable inoculum size (Fig. 1A). The increased virulence of D36/99 was also reflected in faster replication kinetics. D36/99 replicated with a profile similar to that of the two reference strains, whereas replication of D36/95 was significantly delayed (Fig. 1B).

The elevated potential of D36/99 to deplete CD4^+ T lymphocytes was paralleled by its ability to induce apoptosis in CD4^+ T cells. Apoptosis was measured independently by several assays: annexin V binding to phosphatidylserine as a marker for the loss of cell membrane asymmetry (Fig. 2A); activation of caspase-3, a key enzyme of the apoptotic signal transduction pathway (Fig. 2B); and depolarization of the mitochondrial membrane as a marker of the mitochondrial branch of apoptotic signaling (Fig. 2C). To this end, cells were stained with antibodies to CD4 and CD3 along with annexin V-FITC, the membrane-permeable caspase-3 substrate PhiPhiLux-C5-D2, or TMRM and subjected to FACS analysis (6, 29). Loss of TMRM binding to mitochondria is an indicator of the breakdown of the mitochondrial membrane potential (6). Examination by all three methods showed that cultures infected with D36/99 had much higher levels of apoptosis in CD4^+ T cells than those infected with the earlier D36/95 isolate (Fig. 2). However, apoptosis in D36/99-infected cultures was still slightly lower than that observed in cultures infected with the wild-type strain 7/86.

Depletion and apoptosis of CCR5^+ and CCR5^+ CD4^+ T cells. Disease progression in HIV-infected patients has been shown to correlate with a change in coreceptor usage in many,
though not all, individuals (14, 56, 65). Therefore, we addressed the question of whether the increased potential of D36/99 to induce apoptosis and hence deplete CD4\(^+\) T cells was caused by a broadened coreceptor usage. We used flow cytometry to distinguish CD4\(^+\) T cells into CCR5\(^+\) and CCR5\(^-\)/CXCR4\(^+\) subsets as described previously (28, 37, 45, 55). Interestingly, D36/95 depleted the CCR5\(^+\) subset of CD4\(^+\) T cells to an extent comparable to that of both D36/99 and the R5X4 reference strain 7/86 (Fig. 3A). In contrast, while D36/99 and 7/86 also caused marked depletion of the CCR5\(^-\)/CXCR4\(^+\) subset of CD4\(^+\) T cells, the earlier D36/95 isolate and the R5 reference strain 1/85 did not affect this subset significantly (Fig. 3B). Similarly, D36/95 and 1/85 induced apoptosis mainly in CCR5\(^+\) CD4\(^+\) T cells, whereas D36/99 and 7/86 promoted apoptosis equally in both the CCR5\(^+\) and CCR5\(^-\)/CXCR4\(^+\) cellular subsets (Fig. 3C and D). Thus, the effects of D36/95 appeared to be restricted to CCR5\(^+\) CD4\(^+\) T cells, while cytopathic effects of the later D36/99 isolate were broadened to include both CCR5\(^+\) and CCR5\(^-\)/CXCR4\(^+\) T cells.

Coreceptor phenotype of the D36 isolates. To define the coreceptor usage by the two D36 isolates more directly and quantitatively, GHOST cell infection assays were performed. CCR5-expressing GHOST (GHOST-R5) cells and CXCR4-
expressing GHOST (GHOST-X4) cells were infected with D36/95 and D36/99 at an MOI of 0.1 (11). The prototypical strains 49-5 (R5) and NL4-3 (X4) were used as positive controls for CCR5 and CXCR4 usage, respectively (62). As expected, 49-5 exclusively infected GHOST-R5 cells, as indicated by LTR-mediated expression of the fluorescent marker, whereas NL4-3 infected GHOST-X4 cells (Fig. 4A). The residual infection of GHOST-R5 cells by the X4 strain NL4-3 was due to the endogenous low-level expression of CXCR4 in parental GHOST cells. Importantly, both D36/95 and D36/99 infected GHOST-R5 cells to an extent comparable to that of 49-5. In contrast, D36/95 infected GHOST-X4 cells but with a fourfold lower efficiency than D36/99. These results indicate that D36/99 has a significantly higher potential to infect CXCR4-expressing cells than D36/95 (Fig. 4A).

We tested the differential CXCR4 utilization by these viruses further by analyzing their sensitivity to the CXCR4 antagonist AMD3100 (22, 53). Dose-response curves were determined with AMD3100 to inhibit infection of CXCR4-HLa CD4 cells (7, 33) by D36/95, D36/99, and the two reference viruses 49-5 (R5) and NL4-3 (X4). Infection by either 49-5 or D36/95 was not significantly inhibited by AMD3100, confirming their independence from CXCR4 (Fig. 4B). In contrast, infection by NL4-3 or D36/99 was inhibited effectively by AMD3100, with a 50% inhibitory concentration (IC50) of 38 nM and 85 nM, respectively (Fig. 4B). Notably, even at AMD3100 concentrations as high as 2,500 nM, infection with D36/99 could not be inhibited completely, while that by NL4-3 was nearly fully abolished (Fig. 4B). This suggests that D36/99 can use both CCR5 and CXCR4 as a coreceptor. Taken together, these results demonstrate that both D36 isolates can utilize CCR5 as a coreceptor and that D36/99 can also use CXCR4 efficiently.

Next, we analyzed the coreceptor usage of the D36 isolates within the biologically relevant ex vivo lymphoid cell culture system. Lymphoid cultures were pretreated with AMD3100 (250 nM) and infected with equal doses of D36/95 or D36/99. Depletion and apoptosis of CD4+ T cells overall as well as specific depletion of the CCR5+ and CCR5− subsets of CD4+ T cells were determined 12 days after the infection. As expected, the modest depletion (Fig. 5A) and apoptosis (Fig. 5B) induced by D36/95 was not significantly affected by AMD3100. In contrast, both CD4 depletion (Fig. 5A) and apoptosis (Fig. 5B) induced by D36/99 were strongly inhibited by AMD3100 (Fig. 5A and B). Furthermore, analysis of the CCR5+ and CCR5− subsets showed that AMD3100
prevented depletion of CCR5+ CD4+ T cells by D36/99, but not depletion of CCR5+ CD4+ T cells (Fig. 5C). Again, depletion of the CCR5+ subset by D36/95 was unaffected by AMD3100 (Fig. 5C). These results confirm that D36/99 has a broadened coreceptor usage that allows it to infect both CCR5+ and CCR5+ CXCR4+ CD4+ T cells.

Sequence analysis of the gp120 V1/V2 and V3 regions of the D36 isolates. Coreceptor usage is mostly determined by specific amino acid residues in the V1/V2 and V3 loops of the viral envelope protein gp120 (5, 9, 18, 25, 30, 31, 58). In particular, the high charge and low N-glycosylation status of the V3 loop as well as the high N-glycosylation status in V1/V2 have been linked to X4 usage (12, 18, 34, 39, 47, 63). Therefore, we compared the V1/V2 and V3 regions of the two different D36 isolates to analyze whether specific amino acid changes in these domains could be the molecular determinants for the broadened coreceptor usage of D36/99. We did not detect any amino acid mutations in the V3 loop that would be expected to alter its charge or glycosylation status (Fig. 6). However, we found that the later D36/99 isolate contains an additional potential N-glycosylation site at position 161 in the V1/V2 region (Fig. 6). Thus, it appears that the more efficient CXCR4 usage of the later D36/99 isolate may be attributable to an additional N-glycosylation site in the V1/V2 region.

**DISCUSSION**

Evidence from in vivo observations and ex vivo experiments has strongly pointed to a switch in coreceptor usage as an important factor in HIV disease progression. Although the precise mechanism remains unclear, such phenotypic and ge-
In contrast, the increased cytopathicity of the D36/99 isolate animal had completely repaired the infection and AIDS (35, 48). PCR analysis showed that this of the rhesus macaques infected with SIV strains with mutations displayed augmented infectivity and increased downregulation paired (10). Moreover, the virus with this repaired Nef protein long-term nonprogressor infected with an HIV-1 strain with evolution of the viral tropism is an important factor in the disease progression of patient D36 despite the absence of Nef. However, additional mechanisms not delineated here may be involved as well. In conclusion, we have shown that an HIV-1 strain with deletions in the nef gene that were found to be partially repaired (10). Moreover, the virus with this repaired Nef protein displayed augmented infectivity and increased downregulation of CD4 and of MHC class I molecule expression (10). Similarly, rhesus macaques infected with SIV strains with mutations of the nef start codon, as well as deletions and insertions in the coding sequence of nef, were able to express a truncated version of Nef with a reverted start codon after several months of infection (52). Virus isolates with these truncated Nef proteins were much more pathogenic than their parental strains and caused an AIDS-like disease in the macaques (52). Interestingly, vaccination of one of four rhesus macaques with proviral DNA of an SIV strain with the same nef LTR deletion as in the SBBC (SIVSBBC-3) resulted in sustained SIV infection and AIDS (35, 48). PCR analysis showed that this animal had completely repaired the nef LTR deletion (35, 48).

Fig. 6. V1/V2 loop of D36/99 contains an additional potential N-glycosylation site. The top panel shows the gp120 V1/V2 and the bottom panel shows the gp120 V3 loop sequence of both D36 isolates. Potential N-glycosylation sites are printed in bold, and changes between the D36/95 and the D36/99 isolates are highlighted by a box. Amino acid numbers are based on the D36/95 sequence.

V1/V2-loop

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V3-loop

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notypic changes are thought to be the result of an evolutionary selection process (14, 56, 65). The present study reveals that the low level of replication in vivo of an HIV-1 strain with spontaneous deletions in the nef LTR region (Fig. 1B) was sufficient to allow evolution of the viral env gene from a predominantly R5 phenotype toward a predominantly X4 phenotype. The more efficient CXCR4 usage of D36/99 appears to be caused by a single additional N-glycosylation site in the V1/V2 region of the viral envelope gp120. Moreover, such viral evolution seems to have been dictated by the same evolutionary processes that cause changes in typical wild-type nef HIV strains.

Importantly, the change in coreceptor usage was manifested by an increased potential of the virus to infect CCR5+CXCR4+ lymphocytes (Fig. 4) and to deplete and induce apoptosis in this T-cell subset of human lymphoid tissue (Fig. 1A, 3, and 5). Earlier studies had implicated a more promiscuous coreceptor usage and a wider target cell range as a key pathological mechanism underlying disease acceleration following emergence of X4 strains in patients infected with typical wild-type nef strains (14, 56, 65). The increased cytotoxicity of the D36 virus observed in this study strongly suggests that a similar evolution of the viral tropism is an important factor in the disease progression of patient D36 despite the absence of Nef. However, additional mechanisms not delineated here may be involved as well.

Recently, Kirchhoff and coworkers reported a case of a long-term nonprogressor infected with an HIV-1 strain with deletions in the nef gene that were found to be partially repaired (10). Moreover, the virus with this repaired Nef protein displayed augmented infectivity and increased downregulation of CD4 and of MHC class I molecule expression (10). Similarly, rhesus macaques infected with SIV strains with mutations of the nef start codon, as well as deletions and insertions in the coding sequence of nef, were able to express a truncated version of Nef with a reverted start codon after several months of infection (52). Virus isolates with these truncated Nef proteins were much more pathogenic than their parental strains and caused an AIDS-like disease in the macaques (52).

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Live attenuated HIV strains like the one seen in the SBBC have been proposed as a vaccine to prevent infection with wild-type HIV (19). A key challenge in developing a safe, effective attenuated virus vaccine is that a strong immune response seems to depend on the degree of viral replication (51). However, even at a very low level, replication harbors the risk of evolution toward higher virulence. Several reports of spontaneous repair of deletions in the nef gene in vivo resulting in increased virulence and the onset of an AIDS-like disease (21, 52, 64) have challenged the safety of this vaccination concept. Therefore, vaccine candidates have been developed with multiple mutations in accessory genes (20, 27, 44). These second-generation vaccines are thought to be much safer but may still be able to elicit an effective immune protection (32, 44). However, even a triple-deleted SIV strain caused AIDS in neonatal macaques and in a small proportion of adult animals (3, 4). Moreover, the present study shows that the attenuation of a virus with deletions in the nef gene can be overcome not only by repair of the nef gene, but also by compensatory changes in other genes. In particular, the deletions in the D36 virus did not prevent it from evolving to higher virulence by broadening its coreceptor usage. Indeed, in contrast to nef and other accessory genes, env is an essential HIV gene that cannot be deleted to provide a higher level of protection without completely inhibiting replication. The high risk of using a live attenuated virus as a vaccine is further supported by the fact that two more members of the SBBC now have declining CD4 counts and detectable viral loads (8), although the underlying mechanisms in these two cases remain unknown.

In conclusion, we have shown that an HIV-1 strain with multiple deletions in the nef LTR region can still mutate to a higher level of virulence in vivo by widening its coreceptor usage and target cell range. This implies that the low level of replication of an attenuated virus is sufficient to allow substan-
tial viral evolution and shows that the use of live attenuated nef-deleted viruses as a vaccine is not safe.

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