JOURNAL OF VIROLOGY, July 1999, p. 5577–5585 Vol. 73, No. 7
0022-538X/99/$04.00+0

Shift of Clinical Human Immunodeficiency Virus Type 1 Isolates from X4 to R5 and Prevention of Emergence of the Syncytium-Inducing Phenotype by Blockade of CXCR4

JOSÉ A. ESTÉ,[1]* CECILIA CABRERA,[1] JULIÀ BLANCO,[1] ARANTXA GUTIERREZ,[1] GARY BRIDGER,[2] GEOFFREY HENSON,[2] BONAVENTURA CLOTET,[1] DOMINIQUE SCHOLS,[3] AND ERIK DE CLERCQ[3]

Institut de Recerca de la SIDA—Caixa, Retrovirology Laboratory, Hospital Universitari Germans Trias i Pujol, 08916 Badalona, Spain[1]; AnorMED Inc., Langley, British Columbia V2Y 1N5, Canada[2]; and Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium[3]

Received 22 January 1999/Accepted 12 April 1999

The emergence of X4 human immunodeficiency virus type 1 (HIV-1) strains in HIV-1-infected individuals has been associated with $CD4^+$ T-cell depletion, HIV-mediated $CD8^+$ cell apoptosis, and an impaired humoral response. The bicyclam AMD3100, a selective antagonist of CXCR4, selected for the outgrowth of R5 virus after cultivation of mixtures of the laboratory-adapted R5 (BaL) and X4 (NL4-3) HIV strains in the presence of the compound. The addition of AMD3100 to peripheral blood mononuclear cells infected with X4 or R5X4 clinical HIV isolates displaying the syncytium-inducing phenotype resulted in a complete suppression of X4 variants and a concomitant genotypic change in the V2 and V3 loops of the envelope gp120 glycoprotein. The recovered viruses corresponded genotypically and phenotypically to R5 variants in that they could no longer use CXCR4 as coreceptor or induce syncytium formation in MT-2 cells. Furthermore, the phenotype and genotype of a cloned R5 HIV-1 virus converted to those of the R5X4 virus after prolonged culture in lymphoid cells. However, these changes did not occur when the infected cells were cultured in the presence of AMD3100, despite low levels of virus replication. Our findings indicate that selective blockade of the CXCR4 receptor prevents the switch from the less pathogenic R5 HIV to the more pathogenic X4 HIV strains, a process that heralds the onset of AIDS. In this article, we show that it could be possible to redirect the evolution of HIV so as to impede the emergence of X4 strains or to change the phenotype of already-existing X4 isolates to R5.

Human immunodeficiency virus type 1 (HIV-1) strains isolated from newly infected individuals are predominantly macrophage tropic (MT) and non-syncytium inducing (NSI) and require CC-chemokine receptors such as CCR5 as entry cofactors in combination with CD4 (1, 16) (referred to as R5 HIV strains [2]). T-tropic (TT) strains are rapidly replicating, syncytium-inducing (SI) strains that use the CXCR4 receptor (referred to as X4 strains [2]); they appear much later, after the primary infection, and their emergence is associated with a rapid decline of $CD4^+$ T cells that heralds the breakdown of the immune system and the onset of AIDS (9, 16, 19, 32, 33, 35). SI X4 viruses appear to exert their deleterious effect on the immune system not only by direct cytopathic effects on $CD4^+$ T cells but also by the indirect killing of $CD8^+$ T cells that is mediated by CXCR4 (22). Furthermore, it has also been shown that lymphoid cells infected with R5 strains retain their immunocompetence but that, conversely, infection by X4 strains blocks the immune response to specific antigens (20). This implies that the immunodeficiency hallmarking the progression of AIDS is due, at least in part, to the emergence of the more pathogenic SI X4 strains (3). Therefore, it can be inferred that strategies directed to prevent the emergence of X4 strains would be beneficial to HIV-infected individuals.

It has been recently shown that the bicyclam AMD3100 is a highly potent inhibitor of X4 HIV strains, and its mode of action resides in a selective antagonism of CXCR4 (15, 28), the receptor for the CXC-chemokine stromal cell-derived factor 1 (SDF-1) (5). AMD3100 competes with the binding of SDF-1 to its receptor, shuts off the intracellular Ca^{2+} mobilization induced by SDF-1, and does not trigger an intracellular signal by itself. In this article, we show that the evolution of HIV-1 can be directed so as to prevent the emergence of the more pathogenic X4 strains over the less pathogenic R5 strains by blockade of the CXCR4 receptor.

* Corresponding author. Mailing address: Fundació irsiCaixa, Retrovirology Laboratory, Hospital Universitari Germans Trias i Pujol, 08916 Badalona, Spain. Phone: 34-93-4656374. Fax: 34-93-4653968. E-mail: jaeste@ns.hugtip.scs.es.

MATERIALS AND METHODS

Compounds, viruses, and cells. The bicyclam AMD3100 [1,1′-(1,4-phenylenebis(methylene))-bis(1,4,8,11-tetrazacyclotetradecane) octahydrochloride dihydrate] was synthesized at Johnson Matthey as described previously (6). SDF-1α was purchased from R&D Systems (London, United Kingdom). Zidovudine (AZT) was purchased from Sigma (St. Louis, Mo.). The HIV-1 strains NL4-3 and BaL and the $CD4^+$ lymphocytic cell lines SUP-T1 and MT-2 were obtained through the Medical Research Council AIDS reagent program. U87-CD4 cells expressing either CCR5 or CXCR4 were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program.

Determination of viral fitness by replication competition of defined mixtures of viruses. Phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) (10^6 in 1-ml volumes) were infected with 25 ng of a mixture of the HIV strains NL4-3 and BaL (the percentage of each strain being 0, 20, 40, 60, 80, or 100% of the total p24 count) in the presence of AMD3100. The cells were incubated for 24 h and then washed twice in phosphate-buffered saline (PBS) and resuspended in medium containing AMD3100 (1 μg/ml). After a 5-day incubation at 37°C, DNA was isolated from infected cells for DNA sequencing. In similar experiments, PHA-stimulated PBMC infected with a predetermined mixture of 99% NL4-3 and 1% BaL in the absence and presence of AMD3100 (1 μg/ml) were cultured and passaged every 7 days in uninfected PHA-stimulated PBMC. After 28 days in culture, p24 antigen was measured in the culture supernatant and DNA was isolated from infected cells for DNA sequencing.

Virus growth in the presence of AMD3100. PHA-stimulated PBMC were infected with low-passage clinical HIV-1 isolates in the presence of AMD3100. HIV replication was measured (every 7 to 8 days) by a p24 antigen detection method (Coulter). The p24 antigen-positive supernatant was further passaged in fresh PBMC. After four passages (28 days) in the presence of the drug, recovered

virus was used for the phenotype assay in MT-2 cells, and DNA was isolated for PCR amplification, DNA sequencing, and cloning.

In vitro emergence of the SI phenotype. The third variable region (the V3 loop) of the envelope of HIV contains a major neutralization epitope and determinants of cell tropism (23), SI capacity, replication rate (11), and coreceptor use (8). The recombination of a V3 loop DNA sequence corresponding to a R5 strain into the DNA sequence of a X4 strain is sufficient to modify the coreceptor use of the resulting virus from X4 to R5 (8). Furthermore, the phenotype of NSI slow-replicating HIV-1 converts to SI fast-replicating strains after prolonged culture in SUP-T1 cells. Mutations within the V3 loop have been shown to be responsible for the conversion into the SI phenotype (11, 12). Therefore, the evolution of HIV strains from R5 phenotype into the X4 or R5-X4 phenotype can also be monitored by genotypic changes that lead to amino acid changes in the V3 loop. The viral clone 168.1 (11, 12, 24) of the NSI slow-replicating phenotype was cultured in SUP-T1 cells in the absence or presence of AMD3100 (1 μg/ml). Every 5 or 6 days, the numbers of syncytia in the cultured cells were scored, and cells were passaged in fresh medium with or without compound. Once syncytia were scored positive in the untreated sample, the AMD3100 culture was continued for 55 more passages (i.e., until 405 days after the initial infection). DNA was isolated from infected cells for DNA sequencing.

Cloning and phylogenetic analysis of HIV-1 *env*. PCR fragments of the *env* gene from proviral DNA were cloned in the pCR-Script SK(+) cloning vector (Stratagene, La Jolla, Calif.) by following the manufacturer's instructions and the procedure described elsewhere (18). Clones were isolated for DNA sequencing, and phylogenetic analysis was done by the neighbor-joining method using the Clustal X (34) software. Bootstrap resampling was used to assess the strength of support for each branch of the phylogenetic trees.

DNA sequence analysis. The gp120 proviral genome was isolated by PCR amplification of total cellular DNA purified from infected cells. For sequencing of the V3 loop, preparative PCR was performed with 5 to 20 μg of total DNA purified by the QIAGEN blood kit and with 0.1 μg of each of the primers TACAATGTACACATGGAATT and ATTACAGTAGAAAATTCC. Then, a second preparative PCR, which amplifies the V3 loop region of gp120, was done with primers TGGCAGTCTAGCAGAAGAAG and TCTGGGTCCCCTCCTG AGGA. For sequencing of the V2 loop, primers AATTAACCCCACTCTGTG TTAGTTTA and GCTCTCCCTGGTCCCCTCTGG were used for the first PCR and primers AATTAACCCCACTCTGTGTTAGTTTA and TGATACTA CTGGCCTGATTCCA were used for a second preparative PCR. DNA sequencing was performed directly on the purified PCR product following the protocol provided by the ABI PRISM cycle sequencing kit, and sequences were analyzed with an ABI PRISM genetic sequencer. The Navigator and Factura DNA analysis software packages (Perkin-Elmer) were used to identify and quantify ambiguous regions of the DNA sequence that are produced when a mixture of two sequences is detected.

Determination of virus phenotype (MT-2 assay). MT-2 cells were infected with different HIV-1 isolates. Cell cultures were monitored for syncytium formation for up to 14 days postinfection.

Coreceptor use by different clinical isolates. U87-CD4 cells expressing either CCR1, CCR2b, CCR3, CCR5, or CXCR4 (5×10^3) were infected with 10 ng of p24 antigen of the corresponding virus strain and incubated for 24 h. Cells were then washed twice with PBS, and fresh Dulbecco's modified Eagle's medium was added. Cells were incubated for four more days, and p24 antigen in the culture supernatant was measured.

RESULTS

Viral fitness determined by replication competition with defined X4-R5 virus mixtures. The effect of AMD3100 on the replication of mixtures of two laboratory-adapted HIV isolates, the X4 isolate NL4-3 and the R5 isolate BaL, was evaluated based on the sequence of the V3 region of gp120 from proviral DNA isolated from PBMC that had been infected with these virus mixtures. The nucleic acid sequence of a fragment of the HIV-1 V3 region of gp120 from proviral DNA isolated from cells infected with either NL4-3 or BaL or from mixtures of these two virus strains was determined. Proviral DNA sequence determination may serve as a marker of the viral fitness of each strain (21). As expected, the DNA sequence corresponding to either NL4-3 or BaL was found if the cells were infected solely with the NL4-3 or BaL strain, respectively. When the cells were infected with a mixture of these strains, DNA sequence analysis showed that the proviral DNA sequence could not be aligned with either the NL4-3 or the BaL sequence but rather corresponded to a mixture of both sequences (data not shown). Six sample sequencing chromatograms are shown in Fig. 1. DNA sequences (positions 50 to 54) correspond to amino acids I and R/N (amino acids 16 and 17) in the V3 loop of gp120. This region is located before the insertion QR in the V3 loop of NL4-3 and could be aligned in all sequences. As expected, the chromatograms indicated the gradual replacement of BaL (sequence AAAT) by NL4-3 (sequence CCGT) when the NL4-3 level in the input virus was increased. Even at the lowest NL4-3 level tested (20% NL4-3 to 80% BaL), the NL4-3 sequence could be detected. However, in the presence of AMD3100, only the BaL strain was detected in the proviral DNA even at the highest NL4-3/BaL ratio in the infecting virus mixture (80% NL4-3 to 20% BaL) (Fig. 1).

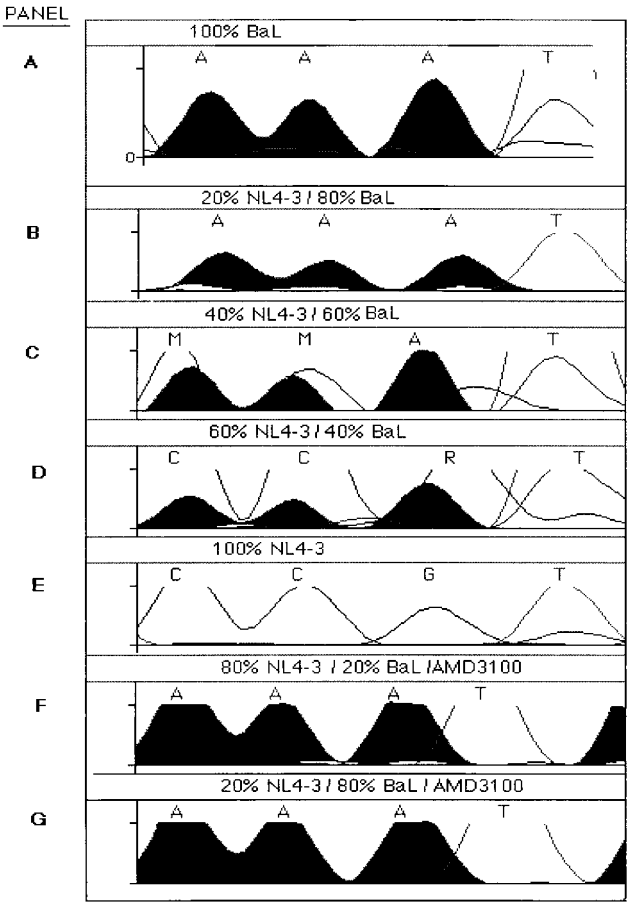

FIG. 1. Determination of viral fitness in a mixed virus population. PHA-stimulated PBMC were infected with either HIV-1 BaL, HIV-1 NL4-3, or a mixture of BaL and NL4-3. At 7 days postinfection, proviral DNA was amplified from infected cells, and the DNA sequence from the V3 loop coding region was obtained and aligned with NL4-3 and BaL proviral sequences. The sample sequencing chromatograms of positions (7144 to 7148 relative to the HXB_2 sequence) in the V3-loop DNA coding region indicate the displacement of BaL proviral DNA by NL4-3 proviral DNA in the infected PBMC. The sequence AAAT (panel A) corresponds to the BaL proviral sequence. Replacement of BaL by NL4-3 can be monitored by the appearance of the CCGT sequence, as indicated by the relative increase in the size of the empty peaks, depending on the ratio of NL4-3 in the infecting virus population (panels B to E). In AMD3100-treated cells, only the BaL sequence emerged, regardless of the proportion of BaL in the infecting virus population (panels F and G). M and R represent the presence of multiple bases in a 50-50% proportion at a given position (M indicates the presence of A or C; R indicates the presence of A or G). The scale of the electropherograms has been reduced to increase sensitivity in the detection of ambiguous (mixture) sequences.

Effect of AMD3100 on the outgrowth of X4 and R5 from X4-R5 virus mixtures. The results presented above indicate

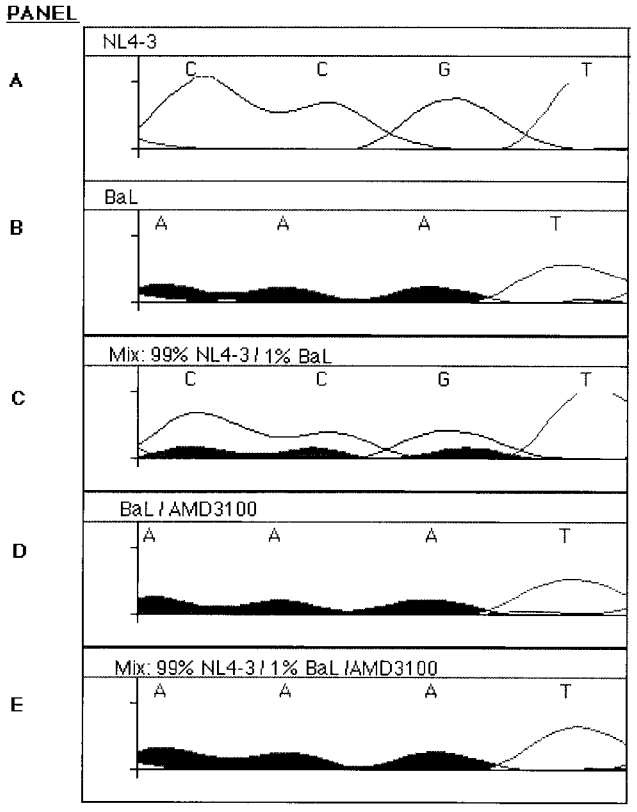

FIG. 2. Selection of the R5 virus after sequential passage of a mixed R5-X4 virus population in the presence of AMD3100. Stimulated PBMC were infected with either HIV-1 NL4-3 or BaL or a mixture thereof comprising 99% NL4-3 and 1% BaL. At 28 days postinfection, proviral DNA was amplified and sequenced. The dominating virus population can be determined by the proportion of the peaks corresponding to the NL4-3 sequence CCG (empty peaks; panel A) or the BaL sequence AAA (filled peaks; panels B and D) in the sample sequence chromatograms. The scale of the electropherograms has been reduced to increase sensitivity in the detection of ambiguous (mixture) sequences. The NL4-3 strain is the dominating virus in untreated cells infected with the 99% NL4-3–1% BaL mixture (panel C). However, the BaL strain became dominant when the virus mixture was exposed to AMD3100 (panel E).

that the replication of the X4 strain NL4-3 is suppressed in the presence of AMD3100. To further assess the influence of AMD3100 on the replicative ability of X4 and R5 virus strains in X4-R5 virus mixtures, a mixture composed of 99% NL4-3 and 1% BaL was used to infect PHA-stimulated PBMC that were then cultured for 28 days (four passages) in the presence or absence of AMD3100. NL4-3 virus replication was inhibited by AMD3100, and NL4-3 proviral DNA became undetectable after 21 days in culture (data not shown). Both NL4-3 and BaL were detectable in the virus progeny at 28 days of an initial virus mixture containing 99% NL4-3 and 1% BaL (as demonstrated by sequencing the V3 loop region of the proviral DNA recovered after 28 days in culture). However, when this virus mixture was cultured for 28 days in the presence of AMD310, only the BaL strain could be recovered (Fig. 2).

Determination of phenotype of clinical HIV isolates grown in the presence of AMD3100. In vivo HIV infection is characterized by the existence of marked heterogeneity in viral populations (25). To better reproduce these conditions, we studied the effect of CXCR4 blockade on the replication of six clinical isolates, three that were defined as SI and three that were defined as NSI in the MT-2 syncytium phenotype assay. PBMC from these six HIV-infected individuals were cocultured with PHA-stimulated PBMC from healthy donors in the presence or absence of AMD3100 (1 μg/ml). After 28 days (four passages) of culture, supernatants were recovered, their viral phenotypes were analyzed by the MT-2 syncytium phenotype assay, and their susceptibilities to AMD3100 and AZT were evaluated. Results are summarized in Table 1. All the NSI strains, grown in the presence or absence of AMD3100, were resistant to AMD3100 but sensitive to AZT. Conversely, the SI strains from untreated cultures showed sensitivity to AMD3100 and AZT, but after growing in the presence of AMD3100, they became insensitive to AMD3100 (50% effective concentration, >1 μg/ml) while remaining sensitive to AZT. Syncytia were observed in MT-2 cells as early as 3 days postinfection when the cells had been inoculated with the SI strains from untreated cultures. However, virus recovered from the cells grown in the presence of AMD3100 did not induce syncytia in MT-2 cells even after 14 days of culture. Similarly, NSI strains also did not induce syncytia. The reference strain NL4-3 scored positive for syncytia in the MT-2 test as early as 3 days postinfection, while the BaL strain remained negative for up to 14 days (data not shown).

Determination of genotype of clinical HIV isolates grown in the presence of AMD3100. Proviral DNA of cells infected with clinical HIV isolates for up to 28 days in the presence or absence of AMD3100 was amplified, and fragments of the *env* gene, corresponding to the V2 and V3 loops, were sequenced. No significant changes in the NSI clinical isolates before and after 28 days of virus replication in the presence of AMD3100

TABLE 1. Low-passage clinical isolates of HIV-1 that were cultured in the presence of AMD3100: phenotype in MT-2 cells and sensitivity to AMD3100 and AZT

Clinical isolate[a]	EC_{50}[b] (μg/ml) following treatment with:		Phenotype in MT-2 cells[c]
	AMD3100	AZT	
NSI strains			
MDM (−)	>1	0.005	−
MDM (+)	>1	0.005	−
MCS (−)	>1	0.004	−
MCS (+)	>1	0.004	−
JGA (−)	>1	0.005	−
JGA (+)	>1	0.02	−
SI strains			
CST (−)	0.13	0.013	+++
CST (+)	>1	0.01	−
AOM (−)	0.11	0.005	+++
AOM (+)	>1	0.006	−
FCP (−)	0.14	0.005	+++
FCP (+)	>1	0.005	−

[a] Clinical isolates or supernatant recovered from PHA-stimulated PBMC of HIV-infected persons with PBMC from negative donors that scored positive for p24 antigen no later than 14 days after the beginning of cocultivation. The cell supernatants were then used for determination of the SI phenotype in MT-2 cells and selection in the presence of AMD3100. The − and + labels indicate strains that were cultured for 28 days in the absence or presence of AMD3100 (1 μg/ml), respectively.

[b] EC_{50}, concentration required to inhibit by 50% the p24 antigen production by HIV-1-infected PBMC: 1×10^6 cells were infected with 10 ng of p24 from each of the clinical isolates in the presence of varying concentrations of the corresponding drug. Four days after infection of cells, one-half (0.5 ml) of the total volume of cell supernatant was removed and an equal volume of fresh medium containing the appropriate drug concentration was added. At day 7, p24 antigen in the supernatant was determined.

[c] MT-2 cells were infected with the corresponding virus isolate. Syncytia were scored microscopically at 14 days postinfection. Scores are expressed as percentages of the wild-type NL4-3 level as follows: 76 to 100% (++++), 51 to 75% (+++), 25 to 50% (++), 1 to 24% (+).

```
Clinical Isolate          V2 loop sequences
/phenotype in MT-2 cells

 CST(-)/SI :  CSFNISTSIRGKVQKEYAFFYKLDIIPIDNDTTSYKLTSC
 CST(+)/NSI:  -----T-G--D-----R-L--R--LV----E-N--RII--

 AOM(-)/SI :  CSFNITKNIGNKKHTEYSLFYKLDVVPIKNDNQSYTLINC
 AOM(+)/NSI:  ------TS------------------------E-------

 FCP(-)/SI :CSFNITTNIRDKVKREHALFYSLDIVKIEDKNETTSYGFNTSYRLRSC
 FCP(+)/NSI:------------M-K-Q------------EN-------L---------

 MDM(-)/NSI:  CSFNISTSIRGKVQKEYAFFYKLDIIPIDNDTTSYKLTSC
 MDM(+)/NSI:  ----------------------------------------

 MCS(-)/NSI:  CSFNVTTSIRDKVQKEYALFYKLDVVPIDNDNNNSYRLINC
 MCS(+)/NSI:  ------------------------------E--.-------

 JGA(-)/NSI:  CSFNITTNLKDKVRNSALFYSLDVVPIENSTSNTSYRLRSC
 JGA(+)/NSI:  ----------------------------------------

                        V3 loop sequences

 CST(-)/SI :  CTRHSHKTRRRIHRGPGRAFYTTGDIIGDMRKAHC
 CST(+)/NSI:  ---L-NN---S-NI---------------I-Q---

 AOM(-)/SI :   CIRPNNNTRRGIHMGPGRTFYAS.DIVGDIRQAHC
 AOM(+)/NSI:  -T---K---KR--I----A---TG--I--------

 FCP(-)/SI :  CTRPNNNTRKGIHIGPGRTFYATGEIIGDIRKAHC
 FCP(+)/NSI:  ----------S-------------------------

 MDM(-)/NSI:  CTRPNNNTRKGIHMGPGKSFYVTDIIGDIRQAHC
 MDM(+)/NSI:  ----------------------------------

 MCS(-)/NSI:  CTRPNNNTRKSINIGPGRAFYATGDIIGDIR Q/K AYC
 MCS(+)/NSI:  ---------R---------------------- K ---

 JGA(-)/NSI:  CCTRPNNNTIKSIHMGSGKAFYLTGQVVGDIRQAHC
 JGA(+)/NSI:  ------------------------------------
```

FIG. 3. Amino acid sequence of the V2 and V3 loop regions of HIV-1 clinical isolates cultured in the presence or absence of AMD3100. Low-passage clinical HIV isolates belonging to the SI phenotype (isolates CST, AOM, and FCP) or the NSI phenotype (isolates MDM, MCS, and JGA) were cultured in PHA-stimulated PBMC for 28 days in the presence (+) or absence (−) of 1 μg of AMD3100/ml. Proviral DNA was isolated from the infected cells and submitted to PCR amplification and DNA sequencing of the V2 and V3 coding regions.

were observed compared to the untreated virus DNA sequences. However, several mutations in the SI strains cultured in AMD3100-treated cells that were not present in the untreated samples were noted. Amino acid changes were found in those V3 loop regions (isolates AOM and CST) and V2 loop regions (isolates AOM and FCP) that have been associated with SI and NSI phenotype and HIV tropism (Fig. 3) (23, 29). PCR products corresponding to the V3 loop sequence were also cloned, and individual clones were sequenced. The consensus sequence derived from the alignment of clone sequences from each virus (data not shown) was identical to the proviral sequence that was determined by sequencing of the amplified pDNA shown in Fig. 3. Figure 4 shows the phylogenetic analyses of the V3 loop amino sequences from two patients' isolates of the SI phenotype that showed changes in the V3 amino acid composition after treatment with AMD3100. Cloned sequences corresponding to the untreated AOM or CST isolates and treated AOM or CST isolates were clustered in separate parts of the tree, indicating a clear shift in the composition of the viral population after treatment with AMD3100. Two clones of the untreated CST isolate clustered together with the treated CST clones, suggesting that the emerging population, although in a minor proportion, was already present in the untreated clinical isolate. Furthermore, sequences from the AMD3100-treated virus clustered together and closer to the V3 sequence of the R5 strain BaL but more distant from the V3 sequence of the X4 strain NL4-3.

Coreceptor use of clinical isolates after culture in AMD3100. As seen in Fig. 5, U87-CD4 cells expressing CCR5 supported the replication of all HIV clinical isolates of the NSI phenotype, as evaluated by p24 antigen production after 5 days postinfection. However, no virus replication was detected in the CXCR4-transfected cells. Conversely, the SI strains were able to infect the CXCR4-transfected cells, and one SI isolate (CST) was able to replicate in both the CXCR4- and CCR5-transfected cells. After being cultured for 28 days in the presence of AMD3100, all the recovered virus strains replicated in CCR5-transfected cells but not in CXCR4-transfected cells regardless of the coreceptor used by the original virus isolate. The replication of the untreated or treated clinical isolates was marginal (<10% of the principal coreceptor used) in U87-CD4 cells expressing CCR1, CCR2b, or CCR3 (data not shown).

Blockade of CXCR4 prevents the emergence of the SI phenotype. Simulating what happens during the course of infection (that is, that R5 strains evolve in some individuals into X4 or R5X4 [dual-tropic] HIV strains), the phenotype of NSI slow-replicating HIV-1 converts to SI fast-replicating strains after prolonged culture in SUP-T1 cells (24, 35). Upon prolonged propagation in SUP-T1 cells, the NSI virus 168.1 tended to give

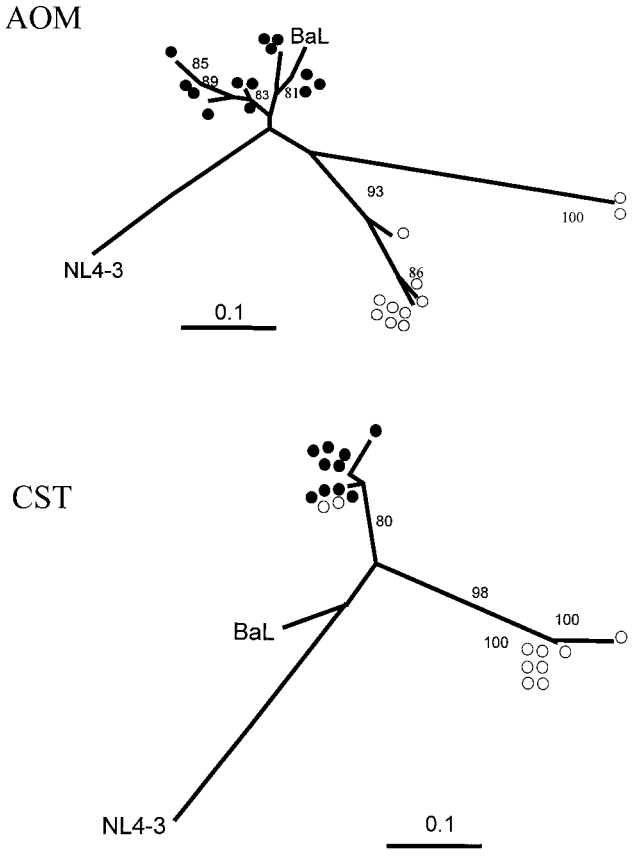

FIG. 4. Unrooted phylogenetic tree analysis of V3 sequence clustering from two HIV-1 isolates of SI phenotype (AOM and CST isolates) cultured in the presence or absence of AMD3100 for 28 days. Phylogenetic analyses of the amino acid sequences were done by the neighbor-joining method with Clustal X software (34). Clones corresponding to the samples from untreated and AMD3100-treated cultures are labeled with empty and filled circles, respectively. The V3 sequences of HIV-1 BaL and NL4-3 were included for comparison. At least 10 clones of each virus were used to construct the phylogenetic trees.

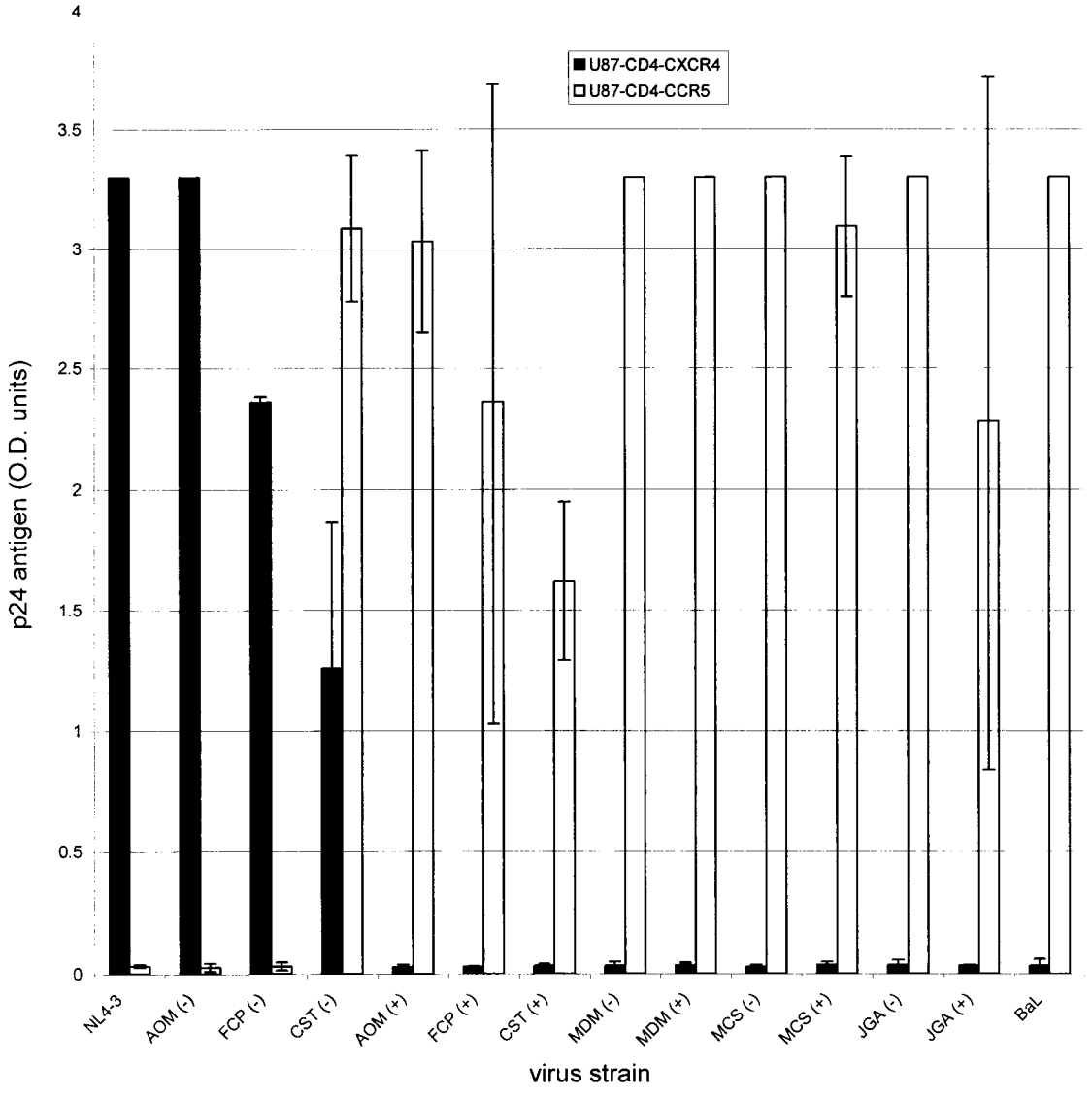

FIG. 5. Coreceptor use of clinical HIV strains after treatment with AMD3100. U87-CD4 cells (5×10^3 cells) expressing CCR5 (empty bars) or CXCR4 (filled bars) were infected with 10 ng of p24 antigen of the HIV-1 strains that were cultured in PBMC for 28 days in the presence (+) or absence (−) of AMD3100 (1 μg/ml). Cells were incubated for 24 h, washed twice in PBS, and resuspended in fresh medium. At 5 days postinfection, p24 antigen was detected in the cell-free supernatant. The phenotype of the parental HIV-1 strains was determined in MT-2 cells (see Table 1)—phenotype SI for isolates CST, AOM, and FCP and phenotype NSI for isolates MDM, MCS, and JGA. The HIV-1 NL4-3 and BaL strains are included for comparison.

rise to virus mutants with an SI phenotype and high replicative capacity. The viral clone 168.1 with an NSI slow-replicating phenotype was cultured in SUP-T1 cells in the absence or presence of AMD3100 (1 μg/ml). Every 4 or 5 days, the numbers of syncytia in the cultured cells were scored, and cells were passaged in fresh medium with or without compound. In the untreated cells, syncytia were first detected after 100 days in culture. At 200 days postinfection, clear cytopathic effect (CPE) and formation of syncytia were noted in the untreated culture. Conversely, no CPE or syncytium formation was detected in the AMD3100 treated cells even after 305 days after the first detection of syncytia in the untreated culture (405 days postinfection), despite low but continuous virus replication (Fig. 6).

To test if genotypic changes paralleled the change from NSI phenotype to SI phenotype, the gp120 proviral genome was isolated by PCR amplification of total cellular DNA purified from infected cells for sequencing of the V3 loop coding region. DNA sequence analysis of proviral DNA isolated from untreated cells where syncytia were observed detected the emergence of mutations in the V3 loop that have been shown to predict the SI phenotype (11, 12, 23) (Fig. 7). Amplified DNA from the untreated cells at day 100 postinfection, when the first syncytia were noted, showed the presence of a mixture of two amino acids, serine (S) or arginine (R), at position 11 of the V3 loop (data not shown). Proviral DNA amplified and sequenced from day 200 postinfection (100 days after the first detection of syncytia) revealed the emergence of mutations at position 6 of the V3 loop, from asparagine (N) to lysine (K); at position 11 from serine (S) to arginine (R); and from glycine (G) to arginine (R) at position 28 of the V3 loop (Fig. 7). Furthermore, there was a net increase in the overall charge of the V3 loop from 3+ to 5+. However, in the culture that was treated with AMD3100, no changes in the

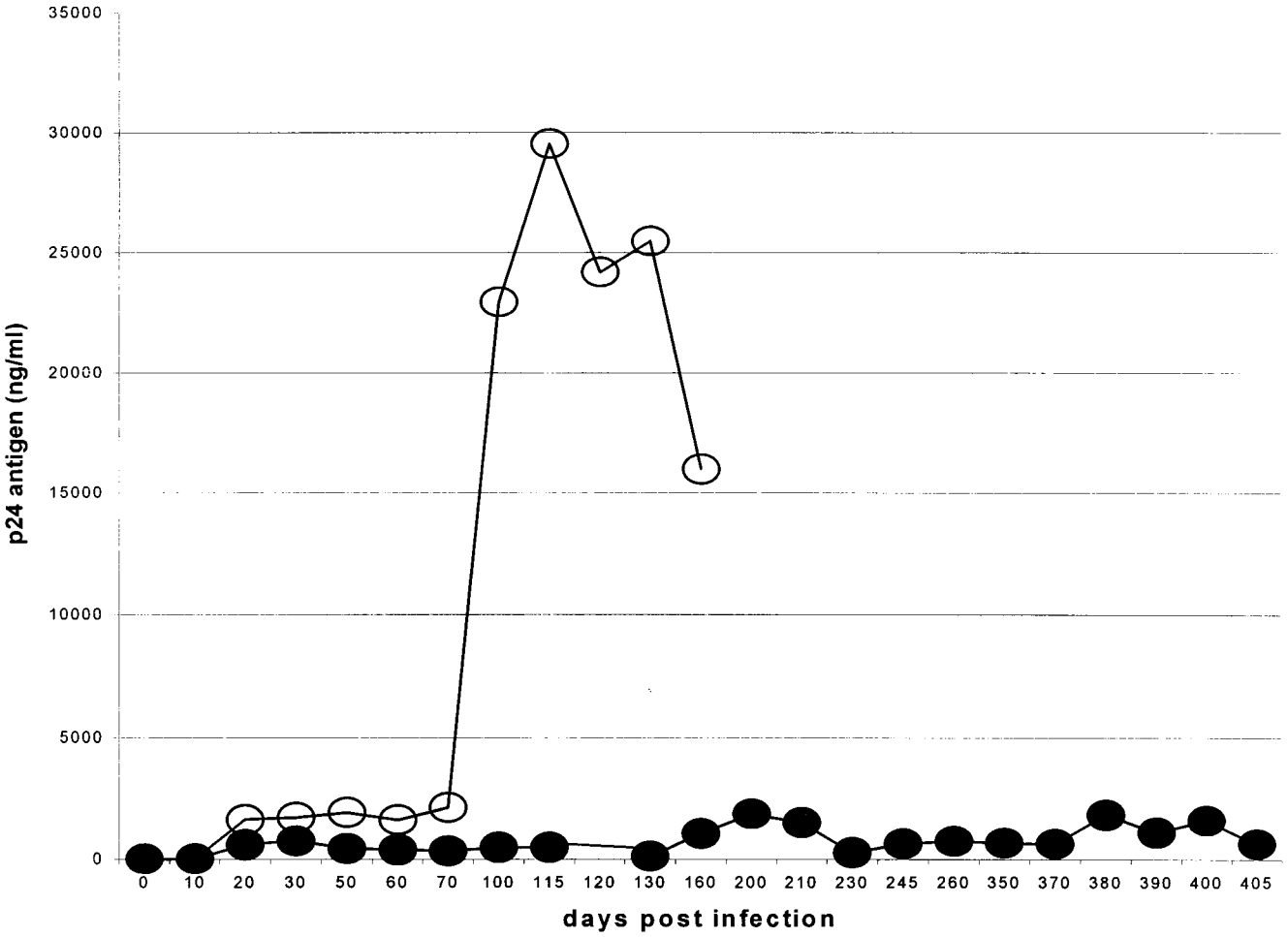

FIG. 6. Replication of NSI strains in SUP-T1 cells in the presence of AMD3100. SUP-T1 cells permit the growth of the HIV-1 168.1. Replication of HIV-1 168.1 was sustained for up to 200 days without AMD3100 (empty circles) or 405 days in the presence of 1 μg of AMD3100/ml (filled circles). Syncytia were noted in the untreated sample after 100 days of culture but were not detected in the treated sample for 405 days.

V3 region of the recovered virus were noted even 305 days after the first detection of syncytia in the untreated culture (i.e., 405 days postinfection).

DISCUSSION

HIV-1 strains isolated from infected individuals are predominantly MT and NSI and mainly use CCR5 as a coreceptor for entry into $CD4^+$ T cells (R5 strains). Over the course of the infection, TT SI variants that use the CXCR4 coreceptor appear (X4 strains). Their emergence has been implicated in $CD4^+$ T-cell decline, $CD8^+$ T-cell apoptosis (22), specific irreversible effects on B-cell activity (19), and the onset of AIDS and disease progression (9, 19, 30, 36).

A major strategy in the fight against AIDS may consist in the prevention of the emergence of the more-pathogenic CXCR4-using strains of HIV. AMD3100 is a potent anti-HIV agent that is targeted at the CXCR4 receptor (15, 28). AMD3100 blocks the intracellular signal induced by SDF-1 but does not induce a signal by itself; thus, it can be considered an antagonist of CXCR4. Its great potency against TT HIV variants (the ratio between the 50% cytotoxic concentration and the 50% effective concentration is >100,000) makes it an ideal candidate to prevent the emergence of X4 strains.

HIV-1 strain	V3 genotype	Charge
168,1/NSI phenotype:	CTRPNNNTRKSIHIGPGRAFYATGDIIGDIRQAHC	3+
168,1/SI phenotype:	-----K----R----------R-..----	5+
168,1/AMD3100:	----------	3+
168,10:	----------R----------T--Q---N------	6+

FIG. 7. V3 loop amino acid sequence of the parental NSI strain (168.1/NSI phenotype) and that obtained from proviral DNA isolated from cells at 200 days (168.1/SI phenotype) and 260 days (168.1/AMD3100) after initiation of the experiment from untreated and AMD3100-treated cells. The amino acid sequence of HIV-1 168.10 strain (11) of SI phenotype is included for comparison. -, homology; ., amino acid deletion.

We have shown in this article that cultivation of a heterogeneous population of HIV, composed of a laboratory-adapted TT (NL4-3) strain and an MT (BaL) strain, in the presence of AMD3100 leads to the selection of the MT over the TT strain (Fig. 1). Even when the initial virus population consisted of only 1% BaL (and 99% NL4-3), BaL completely took over the population after 21 days of subcultivation in PHA-stimulated PBMC. HIV-infected individuals harbor a swarm of closely related viruses, the so-called HIV quasispecies, in which R5 and X4 strains may coexist. From our results, it can be surmised that under selective pressure against the CXCR4 receptor, only MT strains will continue to replicate. That is, in a heterogeneous population, as is the case of a viral population in an infected individual, the fitness of MT quasispecies will be greater than that of TT ones in the presence of AMD3100. Addition of AMD3100 to PBMC from individuals infected with viruses displaying the SI phenotype resulted in a complete block of the SI viruses. Recovered viruses showed reduced sensitivity to AMD3100 and could no longer induce syncytium formation in MT-2 cells. These viruses replicated in CCR5-transfected cells but not in CXCR4-transfected cells. Conversely, the NSI strains remained insensitive to AMD3100 and continued to replicate solely in CCR5-transfected cells. Phylogenetic analysis revealed a drastic change in the viral population upon AMD3100 treatment as predicted from the selection of MT R5 quasispecies. Surprisingly, the clinical isolate FCP (an SI strain) did not show significant changes in the V3 loop region after incubation with AMD3100. However, there were notable changes in the V2 loop coding region which led us to suspect that these changes are responsible for the phenotypic changes observed. The V2 loop genotype has also been associated with the SI-NSI phenotype and HIV tropism (23, 29). Nevertheless, our results clearly show genotypic and phenotypic changes in all treated clinical isolates.

The bicyclam AMD3100 is not active against MT strains of HIV-1 (28). Conversely, AMD3100 was equally active against dual-tropic viruses (which use CCR3, CCR5, CCR8, and CXCR4) (27) in PBMC. More recently, Zhang and Moore (37) have also reported that inhibition of a dual-tropic virus (R5-X4) was inhibited (although only partially) by AMD3100. These results suggest that selection with AMD3100 will favor the emergence of R5 strains over dual-tropic variants. Our results show that only those quasispecies that use CCR5 are allowed to survive while both X4 and R5X4 strains are selected out. That is, AMD3100 exerts selective pressure over both X4 (i.e., the AOM and FCP isolates) and R5-X4 (i.e., the CST isolate) strains. Furthermore, the clinical isolate CST represents a viral population comprising quasispecies that may use CCR5, CXCR4, or both. After selection with AMD3100, the CST isolate seems to replicate less efficiently in CCR5 cells. The replication of both R5 and R5X4 quasispecies may account for the relatively high p24 antigen production in U87-CD4 cells of the untreated CST isolate; however, after treatment with AMD3100, p24 antigen production reflects only the replication of the selected R5 quasispecies.

De Vreese et al. (14) have developed a partially AMD3100-resistant HIV-1 strain that continued to replicate in MT-4 ($CXCR4^+$) cells. This AMD3100-resistant strain was selected from a highly adapted laboratory strain (NL4-3) that deviates from the consensus sequence of primary clinical isolates. Furthermore, the AMD3100-resistant strain was selected in the lymphoid cell line (MT-4) that does not allow replication of MT strains. By slowly increasing the concentration of AMD3100 after subsequent passages, the parental NL4-3 strain accumulated an increasing number of mutations that finally rendered the virus resistant to AMD3100. Conversely, the present results indicate that in a system in which R5 strains are able to replicate at the expense of the X4 strains, the R5 strains take over the population, while the X4 strains vanish. Our results suggest that AMD3100 favored the selection of preexisting quasispecies without the need for ongoing mutations. Under the conditions used, passage of NL4-3 in PHA-stimulated PBMC in the presence of 1 μg of AMD3100/ml resulted in the "knockout" of the NL4-3 virus and proviral DNA-negative cells at 21 days after infection. We postulate that the treatment of AIDS patients with a CXCR4 antagonist may revert the SI-X4-TT phenotype to a less pathogenic phenotype. Suboptimal concentrations of AMD3100 would allow SI-X4 variants to escape the inhibitory activity of AMD3100; nevertheless, many mutations accumulating in the gp120 gene of AMD3100-resistant virus could indicate that resistance may not be easily acquired in vivo (14). Mirroring what happens during the course of infection (that is, that R5 strains evolve in some individuals into X4 or R5X4 [dual-tropic] HIV strains [36]), the phenotype of NSI slow-replicating HIV-1 converts to SI fast-replicating strains after prolonged culture in SUP-T1 cells (11, 12, 24). These viruses are able to efficiently replicate in transformed T-cell lines and to form syncytia when grown in MT-2 cells. HIV-1 isolates 168.1 (NSI) and 168.10 (SI) are sequential isolates obtained from the same asymptomatic individual by coculture of his peripheral blood lymphocytes (PBL) with healthy donor PBL (11, 24). In the T-cell line SUP-T1, the syncytium-inducing capacity of a chimeric HXB-2 virus containing only the V3 region from 168.1 or 168.10 accorded with the phenotype of HIV-1 isolates 168.1 (NSI) and 168.10 (SI) (12). Upon prolonged propagation in SUP-T1 cells, the NSI virus 168.1 tended to give rise to virus mutants with an SI and high replicative capacity. We have confirmed, by detection of mRNA by reverse transcriptase PCR (data not shown), that SUP-T1 cells express low but detectable levels of chemokine receptor CCR5 and high levels of CXCR4 (13), explaining why R5 strains, such as 168.1, can infect this cell line, albeit at a low rate of virus replication. In contrast, X4 strains easily infect and propagate in SUP-T1 cells. In this model, AMD3100 prevented the emergence of the SI phenotype and genotype that is observed in untreated infected cells despite slow but continuous viral replication. No CPE or syncytium formation was detected in the AMD3100-treated cells even after 305 days of the first detection of syncytia (405 days postinfection).

These results further support the notion that CXCR4 antagonism maintains the replication of NSI slowly replicating R5 strains while suppressing the replication of SI rapidly replicating X4 strains. We postulate that treatment of an HIV-positive asymptomatic individual with a CXCR4 antagonist would prolong the asymptomatic phase of its viral infection.

Recent studies by Tachibana et al. (31) and Zou et al. (38) have revealed that mice lacking CXCR4 or SDF-1 expression exhibit hematopoietic and cardiac defects, suggesting that CXCR4 and SDF-1 may play an important role in embryonic development and could have nonredundant functions in adults, thus raising some concerns about the use of CXCR4 antagonists as therapeutic agents against HIV. Furthermore, CXCR4-dependent migration to SDF-1 appears to be essential for human stem cell function in NOD-SCID mice (26). No toxicity was observed after administration of AMD3100 (10 mg/kg of body weight/day b.i.d.) to SCID-hu Thy/Liv mice for 28 days in spite of a significant decrease in HIV viral load in the infected mice (10). Low concentrations of a CXCR4 antagonist could be sufficient to prevent or delay X4 strain emergence without inducing an unwanted effect. Alternatively, other strategies, such as intrakine blockade of CXCR4 on targeted cells (7) or CD4-chemokine receptor pseudotypes

(17), could selectively block the use of CXCR4 in T lymphocytes. Nevertheless, ongoing clinical trials with AMD3100 will have to demonstrate both its safety and efficacy as a chemotherapeutic agent against HIV and AIDS. CXCR4 antagonists could be intended as deterrents for the emergence of X4 strains, more than to decrease viral load levels, which can be effectively achieved by triple drug combinations of reverse transcriptase inhibitors and protease inhibitors (4). The concurrent observations that we have made with both laboratory HIV strains and clinical HIV isolates point to the potential usefulness of CXCR4 antagonists in preventing the switch from R5 to X4 that is generally considered a hallmark of the onset of AIDS and/or the progression of the disease. Our findings also suggest that CCR5-blocking agents might speed the evolution and outgrowth of more pathogenic HIV-1 variants that use CXCR4, thereby accelerating the course of disease. The ability of different HIV-1 strains to use coreceptors in addition to CCR5 or CXCR4 in vitro appears to be irrelevant to their drug sensitivity in primary cells (37). Combinations of both CCR5- and CXCR4-blocking agents could effectively inhibit HIV replication and prevent selection of X4 variants.

ACKNOWLEDGMENTS

We thank Miguel Angel Martinez de la Sierra for help with the phylogenetic analysis.

This work was supported by grants from the Spanish Fondo de Investigación Sanitaria (FIS 98/0868), the Fundació irsiCaixa, the Belgian Geoncerteerde Onderzoeksacties (95/5), and the Fonds voor Wetenschappelijk Onderzoek Vlaanderen (G.0104.98). J.B. is the recipient of an Ajut per a la Reincorporació de Doctors (RED) fellowship from the Generalitat de Catalunya.

REFERENCES

1. **Alkhatib, G., C. Comadiere, C. C. Broder, Y. Feng, P. E. Kennedy, P. M. Murphy, and E. A. Berger.** 1996. CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. Science **272:**1955–1958.
2. **Berger, E. A., R. W. Doms, E. M. Fenyö, B. T. M. Korber, D. R. Litman, J. P. Moore, Q. J. Sattentau, H. Schuitemaker, J. Sodroski, and R. A. Weiss.** 1998. A new classification for HIV-1. Nature **391:**240.
3. **Berkowitz, R. D., S. Alexander, C. Bare, V. Linquist-Stepps, M. Bogan, M. E. Moreno, L. Gibson, E. D. Wieder, J. Kosek, C. A. Stoddart, and J. M. McCune.** 1998. CCR5- and CXCR4-utilizing strains of human immunodeficiency virus type 1 exhibit differential tropism and pathogenesis in vivo. J. Virol. **72:**10108–10117.
4. **BHIVA Guidelines Co-ordinating Committee.** 1997. The British HIV Association guidelines for antiretroviral treatment of HIV seropositive individuals. Lancet **349:**1086–1092.
5. **Bleul, C. C., M. Farzan, H. Choe, C. Parolin, I. Clark-Lewis, J. Sodroski, and T. A. Springer.** 1996. The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. Nature **382:**829–833.
6. **Bridger, G. J., R. Skerlj, D. Thornton, S. Padmanabhan, S. A. Martellucci, G. W. Henson, M. J. Abrams, N. Yamamoto, K. De Vreese, R. Pauwels, and E. De Clercq.** 1995. Synthesis and structure-activity relationships of phenylenebis(methylene)-linked bis-tetraazamacrocycles that inhibit HIV replication. Effects of macrocyclic ring size and substituents on the aromatic linker. J. Med. Chem. **38:**366–378.
7. **Chen, J. D., X. Bai, A. G. Yang, Y. Cong, and S. Y. Chen.** 1997. Inactivation of HIV-1 chemokine co-receptor CXCR4 by a novel intrakine strategy. Nat. Med. **3:**1110–1116.
8. **Choe, H., M. Farzan, Y. Sun, N. Sullivan, B. Rollins, P. D. Ponath, L. Wu, C. R. Mackay, G. La Rosa, W. Newman, N. Gerard, C. Gerard, and J. Sodroski.** 1996. The β-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. Cell **85:**1135–1148.
9. **Connor, R. I., H. Mohri, Y. Cao, and D. D. Ho.** 1993. Increased viral burden and cytopathicity correlate temporally with CD4+ T-lymphocyte decline and clinical progression in human immunodeficiency virus type 1-infected individuals. J. Virol. **67:**1772–1777.
10. **Datema, R., L. Rabin, M. Hincenbergs, M. B. Moreno, S. Warren, V. Linquist, B. Rosenwirth, J. Seifert, and J. M. McCune.** 1996. Antiviral efficacy in vivo of the anti-human immunodeficiency virus bicyclam SDZ SID 791 (JM3100), an inhibitor of infectious cell entry. Antimicrob. Agents Chemother. **40:**750–754.
11. **De Jong, J.-J., J. Goudsmit, W. Keulen, B. Klaver, W. Krone, M. Tersmette, and A. De Ronde.** 1992. Human immunodeficiency virus type 1 clones chimeric for the envelope V3 domain differ in syncytium formation and replication capacity. J. Virol. **66:**757–765.
12. **De Jong, J.-J., A. de Ronde, W. Keulen, M. Tersmette, and J. Goudsmit.** 1992. Minimal requirements for the human immunodeficiency virus type 1 V3 domain to support the syncytium-inducing phenotype: analysis by single amino acid substitution. J. Virol. **66:**6777–6780.
13. **Dejucq, N., G. Simmonds, S. Hibbitts, and P. Clampham.** 1998. Study of co-receptors used by NSI HIV-1 strains that infect CD4+ T-cell lines, MOLT-4 and SupT1, abstr. 11111, p. 4. *In* Conference Record of the 12th World AIDS Conference 1998. Geneva, Switzerland.
14. **De Vreese, K., V. Kofler-Mongold, C. Leutgeb, V. Weber, K. Vermeire, S. Schacht, J. Anné, E. De Clercq, R. Datema, and G. Werner.** 1996. The molecular target of bicyclams, potent inhibitors of human immunodeficiency virus replication. J. Virol. **70:**689–696.
15. **Donzella, G., D. Schols, S. W. Lin, J. A. Esté, K. A. Nagashima, P. J. Maddon, G. P. Allaway, T. P. Sakmar, G. Henson, E. De Clercq, and J. P. Moore.** 1998. AMD3100, a small molecule that interacts with the CXCR4 co-receptor to prevent HIV-1 entry. Nat. Med. **4:**72–77.
16. **D'Souza, M. P., and V. A. Harden.** 1996. Chemokines and HIV-1 second receptors. Confluence of two fields generates optimism in AIDS research. Nat. Med. **2:**1293–1300.
17. **Endres, M. J., S. Jaffer, B. Haggarty, J. D. Turner, B. J. Doranz, P. J. O'Brien, D. L. Kolson, and J. A. Hoxie.** 1997. Targeting of HIV- and SIV-infected cells by CD4-chemokine receptor pseudotypes. Science **278:**1462–1464.
18. **Esté, J. A., D. Schols, K. De Vreese, K. Van Laethem, A.-M. Vandamme, J. Desmyter, and E. De Clercq.** 1997. Resistance pattern of human immunodeficiency virus type 1 to dextran sulfate. Specific interaction of sulfated polysaccharides with the gp120 molecule. Mol. Pharmacol. **52:**98–104.
19. **Fauci, A. S.** 1996. Host factors and the pathogenesis of HIV-induced disease. Nature **384:**529–534.
20. **Glushakova, S., J. C. Grivel, W. Fitzgerald, A. Sylwester, J. Zimmerberg, and L. B. Margolis.** 1998. Evidence for the HIV-1 phenotype switch as a causal factor in acquired immunodeficiency. Nat. Med. **4:**346–349.
21. **Harrigan, P. R., I. Kinghorn, S. Bloor, S. D. Kemp, I. Najera, A. Kohli, and B. Larder.** 1996. Significance of amino acid variation at human immunodeficiency virus type 1 reverse transcriptase residue 210 for zidovudine susceptibility. J. Virol. **70:**5930–5934.
22. **Herbein, G., U. Mahlknecht, F. Batliwalla, P. Gregersen, T. Pappas, J. Butler, W. A. O'Brien, and E. Verdin.** 1998. Apoptosis of $CD8^+$ T cells is mediated by macrophages through interaction of HIV gp120 with chemokine receptor CXCR4. Nature **395:**189–194.
23. **Hwang, S. S., T. J. Boyle, K. Lyerly, and B. R. Cullen.** 1991. Identification of the envelope V3 loop as the primary determinant of cell tropism in HIV-1. Science **253:**71–74.
24. **Kuiken, C., J.-J. de Jong, E. Baan, W. Keulen, M. Tersmette, and J. Goudsmit.** 1992. Evolution of the V3 envelope domain in proviral sequences and isolates of human immunodeficiency virus type 1 during transition of the viral biological phenotype. J. Virol. **66:**4622–4627.
25. **Meyerhans, A., R. Cheynier, J. Albert, M. Seth, S. Kwok, J. Sninsky, L. Morfeldt-Manson, B. Asjo, and S. Wain-Hobson.** 1990. Temporal fluctuations in HIV quasispecies in vivo are not reflected by sequential HIV isolations. Dev. Biol. Stand. **72:**349–353.
26. **Peled, A., I. Petit, O. Kollet, M. Magid, T. Ponomaryov, T. Byk, A. Nagler, H. Ben-Hur, A. Many, L. Shultz, O. Lider, R. Alon, D. Zipori, and T. Lapidot.** 1999. Dependence of human stem cells engraftment and repopulation of NOD/SCID mice on CXCR4. Science **283:**845–848.
27. **Schols, D., G. Bridger, G. Henson, S. Struyf, J. Van Damme, and E. De Clercq.** 1998. Bicyclams: a class of highly potent anti-HIV agents that block viral entry into the cell at the chemokine receptor CXCR4. Antiviral Therapy **3:**13.
28. **Schols, D., S. Struyf, J. Van Damme, J. A. Esté, G. Henson, and E. De Clercq.** 1997. Inhibition of T-tropic HIV strains by selective antagonization of the chemokine receptor CXCR4. J. Exp. Med. **186:**1383–1388.
29. **Shioda, T., J. A. Levy, and C. Cheng-Mayer.** 1991. Macrophage and T cell-line tropisms of HIV-1 are determined by specific regions of the envelope gp120 gene. Nature **349:**167–169.
30. **Spijkerman, I., F. de Wolf, M. Langendam, H. Schuitemaker, and R. Coutinho.** 1998. Emergence of syncytium-inducing human immunodeficiency virus type 1 variants coincides with a transient increase in viral RNA level and is an independent predictor for progression to AIDS. J. Infect. Dis. **178:**397–403.
31. **Tachibana, K., S. Hirota, H. Lizasa, H. Yoshida, K. Kawabata, Y. Kataoka, Y. Kitamura, Y. Matsushima, N. Yoshida, S.-I. Nishikawa, T. Kishimoto, and T. Nagasawa.** 1998. The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. Nature **393:**591–594.
32. **Tersmette, M., R. E. de Goede, B. J. Al, I. N. Winkel, R. A. Gruters, H. T. Cuypers, H. G. Huisman, and F. Miedema.** 1988. Differential syncytium-inducing capacity of human immunodeficiency virus isolates: frequent de-

tection of syncytium-inducing isolates in patients with acquired immunodeficiency virus variants and risk for AIDS and AIDS-related complex. J. Virol. **62:**2026–2032.

33. **Tersmette, M., J. M. A. Lange, R. de Goede, F. de Wolf, J. K. M. Eeftink-Schattenkerk, P. T. Schellekens, R. A. Coutinho, J. G. Huisman, J. Goudsmit, and F. Miedema.** 1989. Association between biological properties of human immunodeficiency virus variants and risk for AIDS and AIDS mortality. Lancet **i:**983–985.
34. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignments through sequence weighting position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
35. **Van't Wout, A. B., H. Blaak, L. J. Ran, M. Brouwer, C. Kuiken, and H. Schuitemaker.** 1998. Evolution of syncytium-inducing and non-syncytium-inducing biological virus clones in relation to replication kinetics during the course of human immunodeficiency virus type 1 infection. J. Virol. **72:**5099–5107.
36. **Xiao, L., D. L. Rudolph, S. M. Owen, T. J. Spira, and R. B. Lal.** 1998. Adaptation to promiscuous usage of CC and CXC-chemokine coreceptors in vivo correlates with HIV-1 disease progression. AIDS **12:**F137–F143.
37. **Zhang, Y.-J., and J. P. Moore.** 1999. Will multiple coreceptors need to be targeted by inhibitors of human immunodeficiency virus type 1 entry? J. Virol. **73:**3443–3448.
38. **Zou, Y.-R., A. H. Kottmann, M. Kuroda, I. Taniuchi, and D. R. Littman.** 1998. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. Nature **393:**595–599.