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Syncytium-Inducing (SI) Phenotype Suppression at Seroconversion after Intramuscular Inoculation of a Non-Syncytium-Inducing/SI Phenotypically Mixed Human Immunodeficiency Virus Population

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Two distinct biological phenotypes of human immunodeficiency virus (HIV) have been described: the non-syncytium-inducing (NSI) phenotype, best characterized by the inability to infect MT-2 cells, and the syncytium-inducing (SI) phenotype, with the ability to infect MT-2 cells. The earliest virus population observed following HIV transmission is generally of the NSI phenotype, even after exposure to inocula of mixed NSI/SI phenotype. In this study, the issue of intrapatient selection of virus phenotype following transmission was addressed by studying two cases of accidental transmission. A comparison of the sequences of the V1-V2 and the V3 coding regions of the envelope gene and the p17 region of the *gag* gene showed that the donor-recipient pairs were tightly clustered in all gene segments, but away from local and published transmission controls. The intrasample variation of the p17 sequence was greater in the recipients and smaller in the donors than that of the V3 region sequence, indicating selection of V3 at transmission. In these transmission cases, the effects of an intravenous inoculation of a small quantity of blood containing predominantly SI V3 sequences (6 of 8 clonal sequences) were compared with those of an intramuscular inoculation of a large quantity of blood containing predominantly NSI viruses (14 of 16 clonal sequences). Both SI and NSI V3 regions were demonstrated to be phenotypic expressions of genetically related viral strains. The inoculation of the predominantly SI virus population resulted in the persistence of an SI virus population in the recipient and a rapid $CD4^+$ T-cell decline. The inoculation of the predominantly NSI population resulted in a selective amplification of SI viruses before seroconversion, followed by a suppression of SI viruses at seroconversion and a rapid decline of $CD4^+$ T-cell numbers. These data suggest that the suppression of SI viruses can be accomplished following the development of HIV-specific immunity and that the ability to suppress SI viruses does not prevent the development of immunodeficiency.

There is evidence that isolates obtained during the early asymptomatic stage of infection and those from patients with AIDS or AIDS-related complex are phenotypically distinct (6, 19, 56). Isolates from the early stages do not induce syncytia in cell culture, are not transmissible to cell lines, and retain a capacity to replicate in primary macrophages. During the later stages of infection, variants may appear that are syncytium inducing (SI), are transmissible to permanent cell lines, and have lost their ability to grow in macrophages (11, 50). The appearance of SI variants is associated with an accelerated $CD4^+$ T-cell decline and a more rapid progression to AIDS (29).

In about 50% of the patients, SI variants appear before AIDS is diagnosed. A virus population with a bulk non-syncytium-inducing (NSI) phenotype is clonally homogeneous, whereas a virus population with a bulk SI phenotype frequently contains a majority of NSI clones (48, 49). The preponderance of NSI viruses throughout the course of infection results in the transmission of NSI variants (27); the transmission of SI viruses is rarely observed (41, 46).

Zhu and coworkers (64) proposed three models (low inoculum, selective amplification, and selective transmission) to explain the observation that seroconverters generally have a relatively homogeneous human immunodeficiency virus type 1 (HIV-1) population, which is typically macrophage tropic and NSI, in spite of the fact that transmitters have a mixture of different viruses with a spectrum of phenotypes. The observation that the transmitted virus represents a minor variant in the blood of the transmitter, irrespective of the route of transmission (61, 62), strongly supports the argument against the first model (a low inoculum). The fact that the level of intrasample sequence variability in the *env* gene (V3 and V4 regions) of seroconverting hemophiliacs is lower than the degree of sequence variability in the *gag* gene supports the hypothesis that selective amplification (the second model) may happen in a new host. However, in cases of sexual transmission in which the donor harbors both phenotypes, the observation that the NSI viruses are typically transmitted rather than the frequently faster-replicating SI variants is at odds with this second model. The third model, which is based on the selective advantage of the transmitted virus in penetrating the mucosal barrier of the new host, is the most plausible of the three. Not only is the

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sequence homogeneity explained, but the phenotypical characteristics of the HIV-1 variants found at seroconversion are also explained. Immediate selection for NSI variants without amplification of SI viruses probably occurs at entry.

The V3 region has many different biological functions. It is an immunodominant region (17, 37) that serves as a major target for isolate-specific neutralizing antibodies (21, 23, 25) and contains epitopes that elicit cytotoxic T-cell responses (9, 54) and helper T-cell responses (10, 43). Moreover, it has been shown to play a role in viral replication (55) and contains determinants of viral tropism (7, 42, 52, 60). The sequence elements contributing to these biological properties are not well defined, except for those for the SI capacity.

In the present study, we examined HIV-1 variants in two donor-recipient pairs at the time of primary infection and of the seroconversion of the recipients. The transmission route was one that bypassed mucosal surfaces. These cases allowed us to study the selective advantages of NSI versus SI viruses that were directly inoculated into the peripheral blood. In one case, blood from a patient with AIDS was accidentally given intravenously (36), and in the other case, the blood from a patient with AIDS was injected intramuscularly. The aim of the present study was to determine whether selective outgrowth of NSI HIV-1 variants occurs even when the virus does not have to penetrate a mucosal barrier. We have analyzed the nucleotide sequences from the V1-V2 and V3 hypervariable regions of the *env* gene and part of the *gag* gene (p17) from genomic viral RNA. The variations within the V3, V4, and *gag* gene regions have been previously studied in several different patients upon transmission (63, 64), but the nature of the V1-V2 region upon transmission has been studied only once, in two seropositive mothers with multiple infected children (35).

MATERIALS AND METHODS

Sera. In the first donor-recipient pair (H→O), the 43-year-old index patient H was a homosexual man who had severe HIV-1-related constitutional symptoms (Centers for Disease Control and Prevention category IVA [CDC IVA]) for several months before the transmission and who died shortly after the incident (36). He had never received zidovudine, and p24 antigen was never detected in his serum. A serum sample obtained 36 days after the transmission was used in this study. Recipient O was a 58-year-old monogamous heterosexual man who had been transferred to the hospital after a partial jejunal resection. He was accidentally given an intravenous injection with a syringe containing a small amount (100 μl) of blood from patient H. Serum samples were sequentially collected during the rest of his life, starting at the day of injection. Thirty days after the transmission, p24 antigen was detected, and after 41 days, antibodies appeared. To study the period of viremia before seroconversion and after the presence of antibodies, serum samples collected at days 30 and 137 were used in this study.

The second transmission case (Y→X) was the result of a criminal action (59). The female victim (X) was given an intramuscular injection, by her ex-partner, of a few milliliters of blood taken from an AIDS patient (Y). Index patient Y (28 years), a homosexual man with CDC IVc1, had been treated with zidovudine for 4 months before the transmission. The p24 state of this patient at the time of transmission is unknown. A serum sample was obtained 138 days after transmission. The female victim (X) was a healthy young woman 34 years of age. Serum samples were collected 21 (X1), 95 (X2), and 159 (X3) days after the injection. The first sample was negative for antibodies but positive for p24 antigen. The other samples, X2 and X3, were positive for antibodies and negative for p24 antigen. Table 1 shows the $CD4^+$ T-cell counts and other markers.

gp120 RT-PCR. Several reverse transcriptases (RTs) were tested for their ability to make large cDNAs (data not shown). Of the RTs tested (avian myeloblastosis virus RT from Boehringer and Promega, avian myeloblastosis virus Gold Label RT from HT Biotechnology, Moloney murine leukemia virus RT from Boehringer, Moloney murine leukemia virus RT RNase H-minus from Promega, and Moloney murine leukemia virus RT RNase H-minus Superscript-I from Gibco BRL), Superscript most efficiently synthesized large cDNA products.

RNA was extracted from 50 μl or 100 μl of serum by the guanidinium isothiocyanate-silica method as described previously (3). Nucleic acids were eluted in 60 μl of sterile H_2O, and 20 μl was used in a reverse transcription reaction. Primer and template RNA were allowed to anneal for 3 min at room temperature in a mixture containing 16 ng of antisense primer (5′GCGCCC, HxB2 positions 7813 to 7819), RT buffer as described by Sellner et al. (51) [67 mM Tris (pH 8.8), 17 mM $(NH_4)_2SO_4$, 1 mM β-mercaptoethanol, 6 μM EDTA, and 0.2 mg of bovine serum albumin (BSA) per ml (Boehringer)], deoxynucleoside triphosphates (dNTPs) (0.5 mM each), and 20 U of RNasin (Promega). After the annealing, 200 U of Superscript RT and $MgCl_2$ (end concentration, 6 mM) was added, and this was followed by incubation for 1 h at 37°C in a total volume of 40 μl. After that, the reaction mixture was heated to 95°C for 5 min and an RNase H treatment was performed for 30 min at 37°C. Half of the RT reaction mix (20 μl) was added to 80 μl of a PCR mixture containing the primers (sense, 5′CTTAGGCATCTCCTATGGC, HxB2 positions 5955 to 5973, and antisense, 5′AGTGCTTCCTGCTGCTCC, HxB2 positions 7793 to 7811), 50 mM Tris (pH 8.3), 20 mM KCl, 0.1 mg of BSA per ml, dNTPs (0.1 mM each), 1 mM $MgCl_2$, and 2 U of *Taq* polymerase (Perkin-Elmer Cetus). After incubation for 5 min at 94°C, the reaction mixture was subjected to 35 cycles of amplification in a type 480 DNA thermal cycler (Perkin-Elmer Cetus). A cycle included denaturation for 15 s at 94°C, annealing for 45 s at 55°C, and extension for 1 min at 72°C. Under the conditions employed, a visible band can be obtained on an agarose gel if the concentration of RNA in the serum is equal to or greater than 10^5 RNA molecules per ml. A nested PCR was performed to obtain enough material for direct sequencing and cloning. Specific V1-V2 and V3 nested PCR products were generated. The nested PCR of the V3 region was performed with the primers 5′KSI and 3′KSI as previously described (18). For the V1-V2 PCR fragments, primers (sense, 5′GAGGATATAATCAGTTTATGGGA, HxB2 positions 6538 to 6592, and antisense, 5′TCAAAGGATACCTTTGGACAIGC, HxB2 positions 6833 to 6855) were used with 2.6 mM $MgCl_2$.

***gag* RT-PCR.** *gag* cDNA was generated with the primer gag-4 (antisense, 5′CATTCTGATAATGCTGAAAACATGGG, HxB2 positions 1296 to 1318) under the conditions for the V3 region described by Mulder-Kampinga et al. (39). The first PCR was performed by adding a PCR mixture containing the primer gag-1 (sense, 5′CATGCGAGAGCGTCAGTATTAAGCGG, HxB2 positions 795 to 817), buffer (identical to the buffer for the gp120 RT-PCR), $MgCl_2$

TABLE 1. Markers and characteristics of two HIV transmission cases

Patient	Days after transmission	p24 antigen (pg/ml)	Antibody response	$CD4^+$ cells (cells/mm^3)	Bulk phenotype[a]	Predicted phenotype[b]	No. of predicted SI clones (total no. of clones)
Donors[c]							
H	36	1	+	ND[d]	SI	SI	6 (8)
Y	138	10	+	40	SI	SI	2 (16)
Recipients							
O1	30	41	−	400	SI	SI	8 (8)
O2	137	0	+	290	SI	SI[e]	ND
X1	21	>100	−	ND	ND	SI	10 (10)
X2	95	ND	+	ND	ND	NSI	0 (10)
X3	159	0	+	180	NSI	NSI[e]	ND

[a] Virus isolated from PBMCs was tested for syncytium induction in MT-2 cells.
[b] Judged on the basis of the amino acid sequences at positions 11 and 25 within the V3 loop.
[c] Both donors had AIDS at the time of transmission.
[d] ND, not determined.
[e] Predicted on the basis of the direct sequence analysis of the V3 PCR product.

(end concentration, 2.0 mM), dNTPs (0.2 mM each), and *Taq* to the RT reaction. For the nested PCR, the primers gag-2 (sense, 5′CATAAGCTTGGGAAA AAATTCGGTTAAGGCC, HxB2 positions 835 to 856) and gag-3 (antisense, 5′CTTCTACTACTTTTACCCATGC, HxB2 positions 1248 to 1268) were used with 1.5 mM $MgCl_2$ and dNTPs (0.2 mM each).

Cloning and sequencing. The PCR products were cloned with the TA cloning kit (Invitrogen, San Diego, Calif.) according to the manufacturer's recommendations. Positive colonies were screened by PCR. Colonies were resuspended in 50 μl of brain heart infusion medium, and 2.5 μl of this suspension was amplified for 25 cycles with the Sp6 primer (5′GATTTAGGTGACATATAG) and T7 primer (5′TAATACGACTCACTATAGGG). Positive PCR products were sequenced with dye-labelled primers (Sp6 and T7) (*Taq* dye primer cycle sequencing kit; Perkin Elmer/Applied Biosystems) according to the manufacturer's PCR protocol for double strands and analyzed with an automatic sequencer.

For direct sequencing, the nested primer pairs were extended with an Sp6 primer sequence for the sense primers and a T7 primer sequence for the antisense primers.

Sequence analysis. Alignment of the sequences was straightforward and performed manually. Gaps were introduced for optimal alignment. For the V3 region, a 282-bp fragment (HxB2 positions 7031 to 7321), including the encoding region of the V3 loop, was analyzed. The V1-V2 envelope region included in this study consisted of 294 bp (HxB2 positions 6562 to 6833), and the $p17^{gag}$ region was 399 bp (HxB2 positions 858 to 1247). Consensus sequences were made by assigning to each position the nucleotide most frequently found in the individual clones of one donor-recipient pair. These consensus sequences were based on 16 to 36 clonal sequences. Intrasample and intersample sequence variations for nucleotide sequences were calculated using Hamming distance (22). Positions at which an alignment gap had to be introduced in one of the sequences were excluded from the calculation (pairwise gap deletion). Phylogenetic analysis was carried out by the neighbor-joining method of the MEGA program (34). The nucleotide distance matrix input for the neighbor-joining program was generated by Kimura's two-parameter estimation (28). Bootstrap resampling (100 replications) was employed to place approximate confidence limits on individual branches.

The sequences were analyzed with sequences obtained from various published proven transmission cases. Consensus sequences (see Fig. 3) were taken from the published control donor-recipients, and individual sequences (e.g., A190-c03) were taken from the donor. In addition, the African A12→A11 sexual transmission case was included to serve as an outgroup to root the phylogenetic tree (61).

Nucleotide sequence accession numbers. The sequences determined in this study were given GenBank accession numbers Z47411 to Z47540.

RESULTS

Analysis of HIV sequences. (i) Intrapatient variations for donors H and Y. The serum samples from H and Y were taken 36 and 138 days after transmission, respectively.

Figure 1 shows the deduced amino acid sequences of all three regions. A consensus sequence was made of all clonal sequences belonging to one donor-recipient pair. All clonal sequences of the donors, independent of the sequenced region, were unique, except for two clonal V3 sequences of donor H (Hc07 and Hc10, represented by Hc07 in Fig. 1).

In addition to nucleotide substitutions resulting in silent and nonsilent mutations, length polymorphism was observed in the V3 region outside the V3 loop, in the V1 region, and in the p17 gene in the Y→X transmission case and in the V1-V2 region in the H→O transmission case. The diversity of nucleotide sequences within each sample was estimated as the mean distance between sequences within a sample, and these variations are shown in Table 2. The substantially greater heterogeneity of sequences observed in the V3 region by comparison with those in the p17 region in chronically infected patients has been documented before (24). The V1-V2 regions of both donors contained two clear subpopulations (Hc12 versus Hc02 and Y1c04 versus Y1c06), resulting in a higher intrasample sequence variation compared with that of the V3 and p17 regions.

(ii) Intrapatient variations for recipients O and X. The clonal sequences obtained from a serum sample of recipient O, collected 30 days after the incident, were completely homogeneous and identical with two clonal sequences of donor H (Fig. 1). Greater variation was observed in the V1-V2 region, while three clonal sequences, represented by O1c03 (Fig. 1B), were identical with one of the donor clonal sequences, Hc12.

The rate of misincorporations introduced during PCR and cloning under the conditions used has been studied previously for the V3 and p17 regions (40). Two independent experiments showed a misincorporation rate of 9 out of 4,140 bp (0.22%) and 8 out of 3,069 bp (0.26%) for the V3 region and 1 out of 3,900 bp (0.03%) and 4 out of 3,510 bp (0.11%) for the p17 region. Taking into account the fact that only random point mutations were found in the *gag* gene of patient O, it is possible that these mutations were introduced in vitro (Fig. 1C). Besides, the observed mutation rate of 0.10% (Table 2) is in agreement with the experimental misincorporation rate. The same argument can be used for the diversity observed in the V1-V2 region. The mean variation in this region is somewhat higher (0.43% for V1-V2 versus 0.10% for p17), and all mutations were unique among the individual clones. Some mutations may represent true variations, however, since the direct sequence confirmed the existence of polymorphism at some of these sites, which were polymorphic in the clonal sequences (data not shown). The homogeneity of the p17 and *env* region sequences early in infection is also described by Zhang et al. (63), who found that two out of the nine individuals with a primary infection showed no variation in either region.

The clonal sequences obtained from samples X1 and X2 were essentially homogeneous in the V3 region of the *env* gene (on the amino acid level, 8 of 10 clonal sequences of X1 and 9 of 10 clonal sequences of X2 were identical), while the p17 and V1-V2 regions showed more sequence heterogeneity. Zhang et al. (63) as well as Zhu et al. (64) have recently shown that the homogeneity is confined to the V3 region and is not always present in the p17 region in primary infected individuals. Our data support these results. Overall, the sequence variations in X1 and X2 were higher than those found in the samples of O, and several heterogenous positions in X1 and X2 were also polymorphic in the sequences from Y. Therefore, these variations must represent actual in vivo variants.

(iii) Interpatient variations. The interpatient variations shown in Table 3 were calculated from the consensus nucleotide sequences. In the transmission case of H→O, the consensus sequences of the donor and the recipient were identical for V3. This was not the case for the V1-V2 and p17 regions. For this region, the nucleotide variation between the consensus sequences was 13.1 and 0.77%, respectively, although identical V1-V2 sequences were observed for H and O. The high V1-V2 intersequence variation is the result of calculating from consensus sequences instead of from each individual sequence. Interestingly, the variations observed for the X1-X2 sequences of all three regions were of the same order of magnitude as that for X1-Y. Clonal sequences of the V3 region and the $p17^{gag}$ region of X2 closely resemble those of the donor Y, while the comparison of the V1-V2 sequences of X2 and Y showed more sequence heterogeneity (the variation for V1-V2 was 5.04%).

The similarity of the V3 sequences of X2 and Y, as observed in Fig. 1 and Table 3, is clearly visible in Fig. 2. The preseroconversion sample, X1, and the sample taken shortly after seroconversion, X2, were very homogeneous by comparison with those from Y. Sequences of Y resembled those of X2 much more than they did those of X1, but sequences closely related to those of X1, with a nucleotide variation of less than 1%, were observed, too.

In both transmission cases, the sequences of the recipient virus matched best with those of the minor rather than the major variants in the serum of the donor, which is in line with the observations of others (47, 62).

Phylogenetic analysis. Many molecular analytical studies have indicated the possibility of an epidemiological linkage (1,

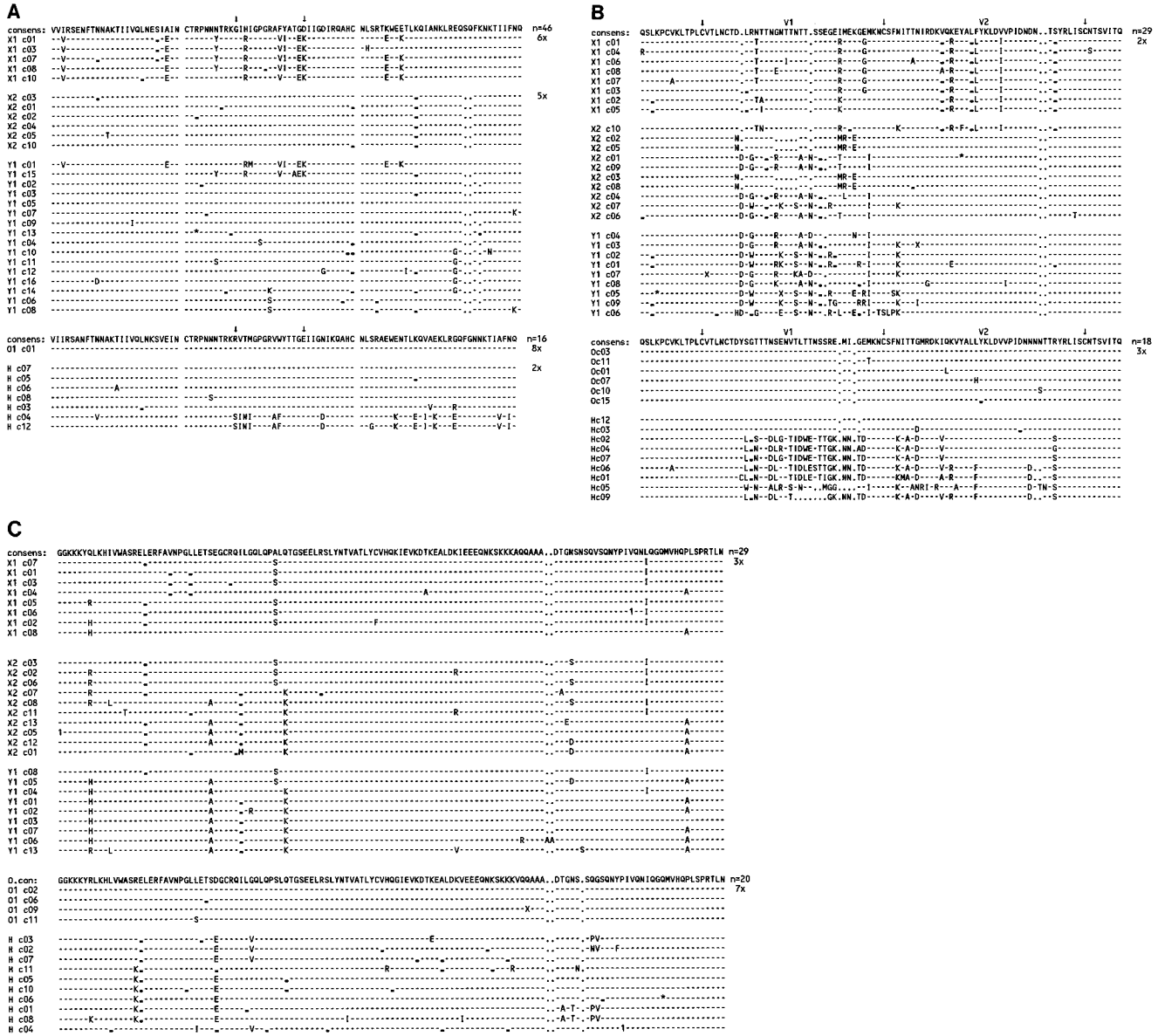

FIG. 1. Deduced amino acid sequences of the V3 (A), V1-V2 (B), and p17 (C) regions. The clonal sequences are aligned against the consensus sequences derived from all clonal sequences obtained from one donor-recipient pair. The number of clones sequenced from each pair is shown at the end of the consensus sequence. The frequency of clones with identical sequences for each sample is given at the end of the clonal sequence. The V3 loop is separated, and the amino acid positions involved in SI capacity are marked (↓). Dashes indicate identity with the reference sequence; dots are introduced to maximize alignment. *, stop codon; ▂, a silent mutation by comparison with the reference sequence; 1, deletion of one nucleotide.

2, 5, 38, 39, 61). The reliability of an epidemiological investigation depends on the selection of the controls. Further, taking into account the fact that the transmitted virus appears to be a minor variant in the donor quasispecies, sequences of independent clones must be used to minimize the chance of missing any critical variant in the donor.

All three sequenced regions described above have been used to support an infection linkage between H→O and Y→X. Unfortunately, however, the number of published background control sequences of the V1-V2 and p17 regions is rather small.

Figure 3 shows the results of the phylogenetic analysis of the V3 sequences by the neighbor-joining method. For this analysis, only published sequences from proven transmission cases were included. Because of the sequence homogeneity of the V3 regions from X1 and X2 (Fig. 1), the consensus sequences rather than the individual sequences of the two different samples were used. The consensus sequence and two divergent

TABLE 2. Intrasample variations

Region	Donor variation (%)		Recipient variation (%)		
	H	Y	O1	X1	X2
V3	3.11	2.34	0	0.76	0.68
V1-V2	6.70	5.24	0.43	1.40	3.40
p17^{gag}	2.44	1.81	0.10	1.51	2.18

TABLE 3. Intersample variations calculated on the basis of consensus sequences

Region	Interpatient variation (%)			
	O1-H	X1-X2	X1-Y	X2-Y
V3	0.00	5.22	4.42	0.80
V1-V2	13.11	3.49	8.53	5.04
$p17^{gag}$	0.77	1.28	2.31	1.02

sequences from Y were included. The sexual transmission case of A12→A11 served as an outgroup in the analysis, because the virus population is of African origin.

The sequences from the donors and recipients in both transmission cases were clustered tightly together (with a bootstrap value of >95% for 100 replicons), making it highly likely that donor Y and recipient X were epidemiologically linked.

The consensus sequences from X1 and X2 were also compared with those from 152 controls from the same geographical area (Amsterdam, The Netherlands). The local controls were direct V3 sequences obtained at the time of seroconversion and were taken from homosexuals, intravenous drug users, and Dutch hemophiliacs (32, 33). The closest local control sequences from among those 152 seroconverters had a similarity of 93.1%, while the Y→X sequences were within a similarity range of from 95.4 to 99.2%, confirming the epidemiological linkage.

In addition, the phylogenetic analysis with the V1-V2 and p17 sequence data set showed the same tight clusters for H→O and Y→X (data not shown).

Suppression of syncytium induction. A good correlation between a positive charge at positions 11 and 25 within the V3 loop, calculated from the first cysteine residue, and the capacity to induce syncytia in culture has been described (15, 20). On the basis of these two positions (11 and 25), all of the clonal sequences from O1 and six of eight clonal sequences from donor H in the H→O transmission case were predicted to be SI variants. In the Y→X transmission case, 10 of 10 clonal sequences from X1 and 2 of 16 clonal sequences from Y were SI variants, while all of the clonal sequences obtained after seroconversion (X2) were NSI variants. Since the X2 sample contained only NSI viruses and the sampling time was 74 days after that of sample X1 (Table 1), which contained only SI clonal sequences, this result clearly shows the suppression of an initially SI virus population by an NSI virus population.

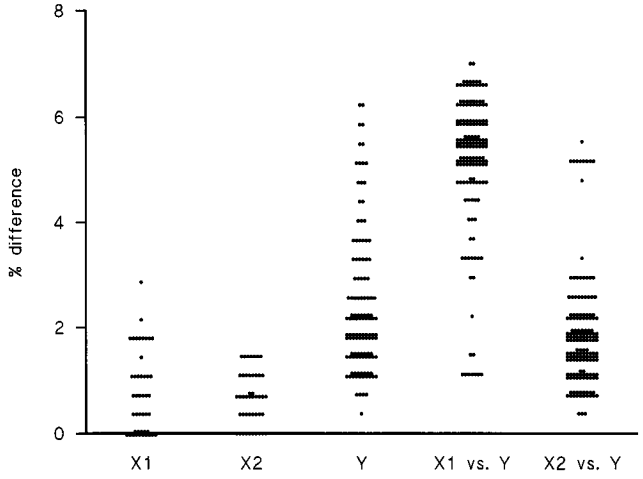

FIG. 2. The pairwise nucleotide distance variations of the clonal V3 sequences of each sample of X and Y are plotted. Each dot represents one pairwise nucleotide distance expressed as a percentage.

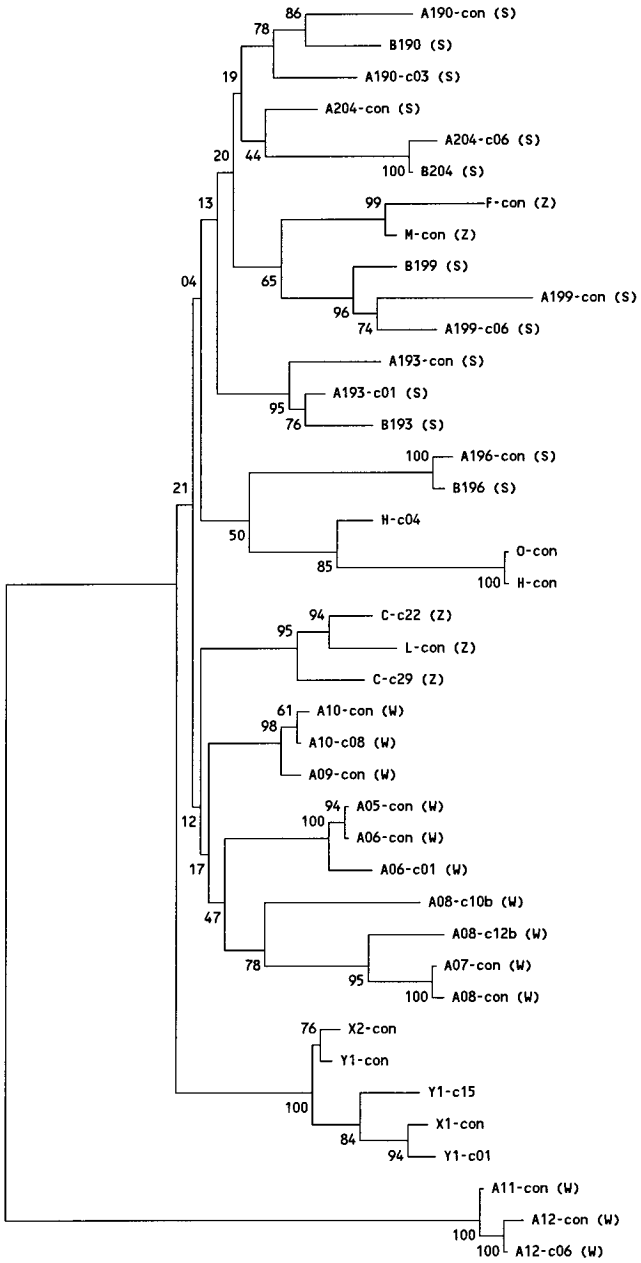

FIG. 3. Results of the phylogenetic analysis of the V3 regions (by the neighbor-joining method part of the MEGA program) from the two studied transmission cases, H and Y, and published sequences from proven transmission cases. The analysis was performed with consensus sequences (con) and divergent clonal sequences of donors (c followed by a number). The donor-recipient pair A12→A11 was of African origin and served as an outgroup. The mean of the bootstrap values obtained for the cluster is given at the root of the cluster. The initials in parentheses refer to the published V3 region sequences. S, Scarlatti et al. (47); W, Wolfs et al. (61); Z, Zhu et al. (64).

The observed suppression of the SI population in the serum of patient X was confirmed by direct sequencing (Table 1) with serum samples obtained 159 and 310 days after transmission. The persistence of an SI population after seroconversion in patient O was analyzed with a serum sample obtained 137 days

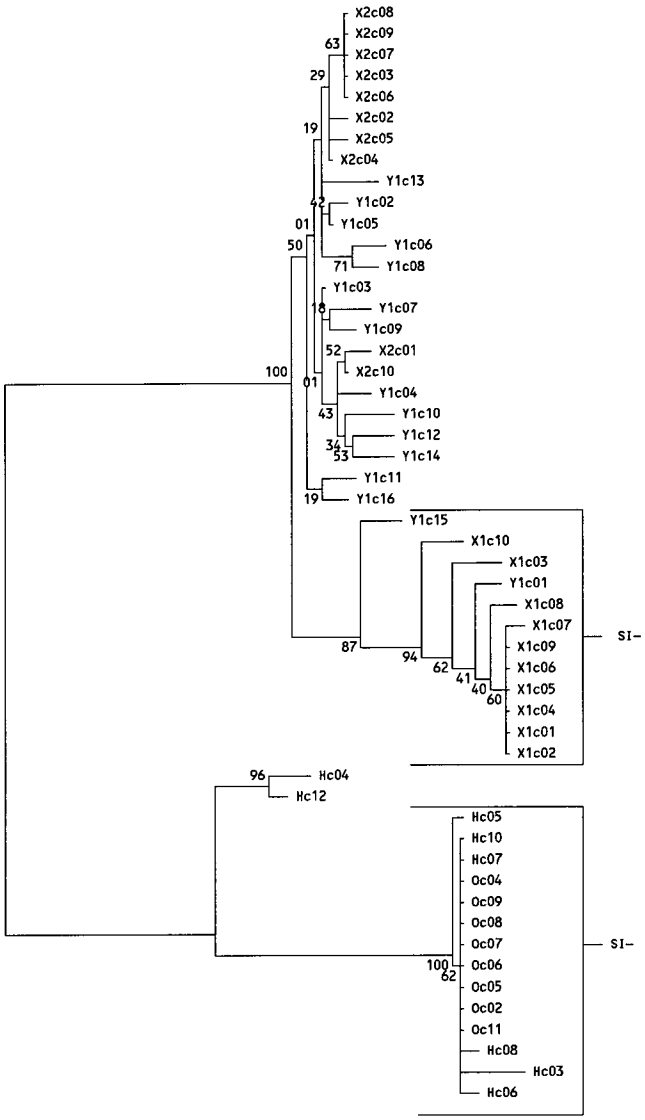

FIG. 4. Results of the phylogenetic analysis of the clonal V3 sequences from the two transmission cases H→O and Y→X. The neighbor-joining method from the MEGA program was used. Clonal sequences with an SI capacity are marked.

after transmission. The V3 direct sequence analysis showed a predicted sequence for a homogeneous SI population (data not shown), which indicated that suppression was not observed in serum of patient O.

All sequences derived for V3 were analyzed by the neighbor-joining method, and the results of this analysis are shown in Fig. 4. The clonal sequences for one transmission case were clustered tightly together, confirming the results of the linkage study (Fig. 3). The clonal sequences from X2 were more closely related to the clonal sequences from Y than they were to the clonal sequences from X1, except for the sequences Y1c15 and Y1c01, which clustered with the sequences from X1. In the H→O transmission case, most of the sequences from donor H were clustered with those from recipient O1. When the predicted phenotype of the V3 clonal sequences is included in Fig. 4, then the variants for which syncytium induction was predicted clearly separate from those for which a lack of syncytium induction was predicted. The high bootstrap values (87 and 100%) strongly suggested that the NSI and SI groups represent true monophyletic groups in both transmission cases. But the NSI and SI variants of one patient were genetically the same virus strains, since no mixing of epidemiologically unrelated sequences was observed (Fig. 3).

DISCUSSION

In this study, we have determined sequences from the virus populations present in the interval between exposure and seroconversion and immediately after seroconversion in two rare transmission cases. In one case, a few milliliters of blood from an AIDS patient was injected intramuscularly (59). In the other case, the recipient was given an intravenous injection of blood (ca. 100 μl) from another AIDS patient (36). In addition to the differences in the amounts of blood and the routes of infection, there were significant differences in the ratios of NSI and SI viruses. In the H→O case, the donor blood sample contained predominantly SI (6 of 8 clonal sequences) viruses, while in the Y→X case, the donor blood contained predominantly NSI (14 of 16 clonal sequences) viruses. The intravenous inoculation of predominantly SI viruses resulted in the persistence of an SI virus population in serum, which was confirmed by the direct sequencing of a serum sample 137 days after transmission. The intramuscular inoculation of a predominantly NSI population resulted in an outgrowth of SI viruses before seroconversion, followed by the suppression of SI viruses after seroconversion.

The two sexual partners described in a somewhat analogous study by Zhu et al. (64) were infected by chronically infected partners and became patients with an acute, self-limited symptomatic illness. From these data, Zhu et al. proposed that the loss of SI viruses after exposure was likely due to inefficient transmission at the mucosal surface. This conclusion is somewhat contradicted by our data, which suggest that SI viruses are transmitted, amplified, and subsequently replaced by NSI viruses. This might be explained by an initial positive selection for SI viruses followed by negative selection against these viruses. The study by Zhu et al., however, analyzed cases of sexual (mucosal) transmission, whereas the results presented here are based on direct, nonmucosal transmissions. Altogether, the published data by Zhu et al. and our data indicate that there are likely to be a number of factors that determine which viruses are transmitted from a donor and which of them then replicate and subsequently persist.

In two sexual transmission studies (61, 64), serum or blood samples from recipients with identified partners were obtained after evident seroconversion, which is comparable to the situation for sample X2 in this study. Although one of the partners in these studies had a mixture of different viruses, a suppression of the transmitted SI virus could have been missed. In another study (63), one of the five primary infections could be predicted to have an SI phenotype. Unfortunately, no sequential samples were analyzed, so the outgrowth of possible NSI viruses and the suppression of this SI variant cannot be confirmed or ruled out. Patient O may be one of the rare cases of the transmission of persistent SI viruses, as reported by Nielsen et al. (41).

Direct sequence analysis was used to confirm the persistence of suppression of a major SI population. Direct sequence analysis of serum sample X2 did not show any polymorphic sites, a result which was supported by the clonal sequences (Fig. 1A). Therefore, less than 10% of the circulating genomic RNA in the serum of patient X after seroconversion could have had an SI phenotype. To evaluate this hypothesis, the heteroduplex tracking assay was developed (16). This technique can be used

to track specific sequence variants within individuals even if these variants constitute between 2 and 5% of the population. Preliminary results obtained with the X2 serum sample showed the presence of SI variants among the major NSI variants. Although SI variants may still be present at low levels, these variants are nevertheless suppressed 310 days after transmission (data not shown).

This study reports on the circulating genomic viral RNA population in the blood. A previous report discussed the finding that after seroconversion, RNA type sequences can persist as a (minority) component of proviral sequences in peripheral blood mononuclear cells (PBMCs) several years after primary infection (53). PBMC samples from both transmission cases were used to study the genotypic and phenotypic characteristics of the transmitted virus population (58). Bulk isolates confirmed the persistence of an SI population in recipient O and the replacement of SI viruses by NSI viruses in recipient X. However, the biological cloning of viruses from the same PBMC samples of patient X showed the presence of SI viruses at a low frequency (5 of the 34 clones), while SI viruses (10 of the 10 clones) were present at a high frequency in the virus population from the PBMCs from the O1 sample from patient O. Interestingly, virus variants present shortly after infection of recipient O were macrophage-tropic SI clones (49). After seroconversion (the O2 sample in Table 1), all the clones still had an SI capacity, but they shifted to become more T-cell-tropic viruses. The V3 loop amino acid sequences (58) derived from the biological NSI clones ($n = 29$) of patient X were identical with the V3 loop amino acid sequences of serum sample X2, and two of the five SI clones had amino acid sequences identical to those found in X1. These results confirm the predictive value of SI capacity as judged by charged amino acids at positions 11 and 25 in V3 and the suppression of SI viruses in patient X. The finding of only SI biological clones in the cells of patient O confirmed the persistence of the SI phenotype.

Phylogenetic analysis showed clear clusterings of the X1 sequences and the SI sequences of Y and of the X2 sequences and the NSI sequences of Y. This indicates that multiple HIV-1 variants have been transmitted. It is unlikely that this can be explained by convergent evolution, because there is no evidence that distinct HIV-1 genotypes (and phenotypes) such as those identified in the X and Y samples in our study can evolve independently within weeks. It is therefore most likely that viruses with different phenotypes were transmitted and that the selection and outgrowth of viruses are host dependent. The genetic relation between the NSI and SI variants in patient X has been made clear in the phylogenetic analysis (Fig. 3).

Although the variants found in X and Y are related, there is a very strict separation within this family between the NSI and SI variants. This conclusion is supported by the high bootstrap value found in a phylogenetic analysis of these sequences. This NSI/SI separation has been reported before (31). The phenomenon probably indicates that the NSI and SI variants represent different populations. The paucity of variants that are intermediate between NSI and SI suggests that such intermediate variants have a fitness disadvantage; only the starting point and the end point of the route, the NSI and SI variants, appear to be fit enough to reproduce high copy numbers.

In both transmission cases, selection due to the mucosal barrier can be excluded. Nonetheless, selection was observed, since a minor population in the donors became the major observed variant in the recipients. In the H→O transmission case, a selective transmission of one genotype can be assumed. The fact that the V1-V2 variants of O1 represent a minor variant of H, together with the observed homogeneity in the V3 and p17 sequences, supports the hypothesis that there is a selective advantage for this variant in the new host.

It has been assumed that the duration of the asymptomatic period is mainly determined by the capacity of the immune system to suppress high-replicating HIV variants (57). Once the immune system is sufficiently attenuated, overt replication of high-replicating HIV variants is thought to result in $CD4^+$ T-cell depletion and the development of AIDS. Patient X was able to suppress the SI virus population, but the $CD4^+$ T-cell count remained severely depressed ($CD4^+$ T-cell count, ≤ 200 cells per mm^3) during one year of follow-up. $CD4^+$ T-cell and $CD8^+$ T-cell counts were only available after 113 days after transmission, so a transient $CD4^+$ and $CD8^+$ T lymphocytopenia often described to occur about the time of seroconversion could have occurred (12, 13). In patient O, this transient lymphocytopenia has been documented (46). After seroconversion, $CD8^+$ T-cell numbers recovered completely, while $CD4^+$ T-cell numbers recovered only partially for 17 days and then rapidly declined again to ≤ 330 cells per mm^3. Taken together, these data show no beneficial effect on $CD4^+$ T-cell numbers whether viruses with the SI phenotype are suppressed or not.

Acute infection with HIV-1 is associated with high levels of virus replication (8, 14, 26, 45). These high titers decline over the weeks following infection, coinciding with the resolution of symptoms (8, 14). This suggests that an efficient immune response is present during the acute phase of infection. Koup et al. (30) have shown that the rapid decline is temporally correlated with the occurrence of cytotoxic T lymphocyte (CTL) responses. The CTL response is the first virus-specific immune response, whereas the appearance of neutralizing antibodies occurs only after virus levels have declined. Recently, other studies have shown that HIV-1-specific CTL activity is a major component of the host immune response associated with the control of virus replication following primary infection (4, 44). It is interesting to speculate that the suppression of the SI variant observed in patient X is due to a strong CTL response against this variant. Consistent with this hypothesis, patient X showed a short-lived increase in the $CD8^+$ cell count (1,200 $CD8^+$ cells per mm^3 113 days after transmission and 800 cells per mm^3 144 days after transmission). However, the role of the infection route in this transmission case is unknown. It is clear that both cell-free viruses and infected cells may have entered the body, and either could have initiated the infection.

These data suggest that the suppression of SI viruses can be accomplished following the development of HIV-specific immunity and that the ability to suppress SI viruses does not prevent the development of immunodeficiency.

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