JOURNAL OF VIROLOGY, Apr. 1991, p. 1968–1975
0022-538X/91/041968-08$02.00/0
Copyright © 1991, American Society for Microbiology
Vol. 65, No. 4

Phenotypic Heterogeneity in a Panel of Infectious Molecular Human Immunodeficiency Virus Type 1 Clones Derived from a Single Individual

MARTIJN GROENINK,[1] RON A. M. FOUCHIER,[1] RUUD E. Y. DE GOEDE,[1] FRANK DE WOLF,[2,3] ROB A. GRUTERS,[1] H. THEO M. CUYPERS,[1] HAN G. HUISMAN,[1] AND MATTHIJS TERSMETTE[1]*

Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory for Experimental and Clinical Immunology of the University of Amsterdam,[1] Municipal Health Center,[2] and Department of Virology, Academic Medical Center,[3] 1066 CX Amsterdam, The Netherlands

Received 21 August 1990/Accepted 17 January 1991

Previously, we and others have demonstrated a relation between the clinical course of human immunodeficiency virus type 1 (HIV-1) infection and biological properties of HIV-1 variants such as replication rate, syncytium-inducing (SI) capacity, and cytotropism. For the molecular analysis of the biological variability in these properties, we generated a panel of phenotypically distinct yet genetically highly homologous infectious molecular clones. These clones were derived from HIV-1 isolates, mostly recovered by direct clonal isolation, from a single individual in whom a transition from non-SI to SI isolates had been identified over time. Of 17 molecular clones tested, 8 were infectious. The clones exhibited differences in SI capacity and T-cell line tropism. Their phenotypes corresponded to those of their parental isolates, formally demonstrating that biological variability of HIV-1 isolates can be attributed to single molecular clones. With these clones we demonstrated that SI capacity and tropism for the H9 T-cell line, almost invariably coupled in primary HIV-1 isolates, are discernible properties. Also different requirements appeared to exist for H9 and Sup T1 cell line tropism. We obtained evidence that T-cell line tropism is not caused by differences in level of HIV-1 expression but most probably is restricted at the level of virus entry. Restriction mapping of four clones with divergent phenotypes revealed a high degree of nucleotide sequence homology (over 96.3%), indicating the usefulness of these clones for the tracking of genetic variability critical for differences in biological phenotype.

In recent years, increasing attention has been given to the biological variability of human immunodeficiency virus type 1 (HIV-1) isolates and the possible importance of this variability for the pathogenesis of AIDS (2, 9, 34). In previous longitudinal studies, we demonstrated that the majority of HIV-1 isolates recovered from asymptomatic individuals are non-syncytium inducing (NSI) in peripheral blood lymphocyte (PBL) culture and do not replicate in the H9 T-cell line. In stable asymptomatic persons, only NSI HIV-1 isolates with low replication rates were found. In contrast, the emergence of syncytium-inducing (SI), H9-tropic viruses invariably heralded a rapid loss of $CD4^+$ T cells and progression to AIDS within 1/2 to 2 years (35, 36).

Recent reports demonstrating a much higher frequency of infected peripheral blood leukocytes in infected individuals than estimated previously have made $CD4^+$ T-cell depletion caused by direct viral killing of $CD4^+$ cells more plausible (17, 29, 32). Indeed, the in vitro properties of SI variants (syncytium induction, high infectivity, and broad host range) all could very well contribute to effective $CD4^+$ T-cell depletion in vivo. On the other hand, our findings that only NSI variants are found in stable asymptomatic individuals and that, compared with SI variants, these viruses are much more monocytotropic indicate that NSI variants are important for the maintenance of HIV-1 infection, putatively because of an increased capacity to evade the immune system (30).

Since biological variability of HIV-1 isolates thus may well be of direct pathogenetic relevance, molecular analysis to identify the genomic elements responsible for these variable biological properties may contribute to a rational design of both therapeutic approaches and vaccine strategies. For this analysis, we wanted to obtain a panel of naturally occurring infectious molecular HIV-1 clones as closely related as possible yet with different biological phenotypes. Therefore, we selected an individual who developed AIDS within half a year after seroconversion and for whom a change in the biological phenotype of his sequential HIV-1 isolates had been observed. From sequential isolates from this individual, mostly obtained by direct clonal isolation, we generated a panel of full-length molecular clones. Studies of the biological characteristics of these clones in PBLs and T-cell lines demonstrated a variety of distinct biological phenotypes. Restriction mapping and sequence analysis of the *tat* gene of four HIV-1 clones with divergent biological properties demonstrated a high degree of genetic homology.

MATERIALS AND METHODS

Virus isolation. HIV-1 was recovered from cryopreserved, sequentially obtained peripheral blood mononuclear cells (PBMCs) from an individual participating in the Amsterdam cohort study on the natural course of HIV-1 infection (11). $CD4^+$ T-cell numbers and HIV-1 p24 antigen levels in serum of this individual were determined as described before (11). PBMCs of the infected individual were cocultivated with 2-day phytohemagglutinin (PHA)-prestimulated PBLs from seronegative blood donors and cultured in the presence of partially purified interleukin 2. Three times a week, the cultures were observed for syncytium formation (34). Twice a week, supernatant samples were obtained for testing in an

* Corresponding author.

TABLE 1. Sequential clinical and virological data for the individual studied

Time point (wk after seroconversion)	Clinical status[a]	No. of $CD4^+$ cells (10^9/liter)	HIV-1 p24 antigen in serum	HIV-1 isolate	Syncytia in PBLs[b]	Transmission to T-cell lines[c]	
						H9	Sup T1
1 (0)	II	0.2	−	320.1	+	+	(+)
2 (6)	II	0.5	−	320.2	−	(+)	(+)
2A (9)	II	NT[d]	NT	320.2A	±	NT	(+)
3 (12)	II	0.3	+	320.3	+	(+)	+
4 (25)	IV.C1	0.2	+	320.4	+	+	+
5 (30)	IV.C1	0.1	+	320.5	+	+	+
6 (49)	IV.C1	0.02	+	320.6	+	+	+

[a] Centers for Disease Control classification.
[b] −, No syncytia observed; ±, occasional small syncytia; +, syncytia induced.
[c] (+), Virus transmissible only upon repeated attempts and prolonged culture; +, virus readily transmissible.
[d] NT, Not tested.

HIV-1 p24 antigen capture enzyme immunoassay (37). Once a week, fresh, 2-day PHA-prestimulated PBLs from a seronegative blood donor were added to the cultures. Transmission of these HIV-1 isolates to the H9 and Sup T1 T-cell lines was attempted by cocultivation with infected PBLs as described before (34).

At time point 4 (see Table 1), an (oligo)clonal isolate was obtained by direct limiting-dilution virus isolation. At time point 2A, biological clonal or oligoclonal HIV-1 isolates were recovered by adapting the HIV-1 isolation protocol for 96-well microtiter plates. Ten thousand patient PBLs per well were cocultivated with 2-day PHA-prestimulated donor PBLs at a 1:10 ratio in the same medium used for bulk virus isolation (final volume, 200 μl). Once a week, culture supernatant was harvested for testing in the p24 antigen capture enzyme immunoassay. Half of the cells were cocultivated in microtiter plates with Sup T1 cells. Syncytium formation in the Sup T1 cell assay was scored on days 1 and 3 by two observers working independently. PBL cultures were kept for 3 to 4 weeks. PBL cultures containing HIV-1 clones of interest were cryopreserved after expansion in T25 flasks. The stability of the biological phenotype of these clones was established in repeated experiments as described before (34).

Molecular cloning of proviral DNA. Essentially, standard techniques described by Silhavy et al. were followed (31). High-molecular-weight genomic DNA was isolated from HIV-1-infected PBLs, with the exception of isolate 320.3, in which case high-molecular-weight DNA was obtained from infected H9 cells. Since the proviral genomes contained no *Xba*I restriction endonuclease sites, the high-molecular-weight DNA was digested to completion with the *Xba*I restriction enzyme. After digestion, the *Xba*I DNA fragments were size fractionated on a linear 10 to 40% sucrose density gradient. DNA fragments of 10 to 20 kb were pooled and ligated to *Xba*I arms of λGEM-11 (Promega), and packaged in vitro by using Promega's Packagene system. HIV-1 DNA-containing bacteriophage λ plaques were identified by plaque hybridization with a ^{32}P-labeled HIV-1 DNA probe, an HIV–lymphadenopathy-associated virus *Hin*dIII fragment (bases 1704 to 8170) in pSP65 (courtesy of M. Martin, National Institutes of Health, Bethesda, Md.). HIV-1-positive clones were obtained by repeated plaque purification. Insert DNA was subcloned into the *Xba*I site of pUC19 and propagated in *Escherichia coli* DH5α (Bethesda Research Laboratories).

Physical mapping and DNA sequence analysis. Physical maps were constructed by multiple endonuclease digestion and a partial endonuclease digestion method described previously (3). Analysis of nucleotide sequence variation among HIV-1 clones was performed according to the method of Saag et al. (26). The sequence analysis of the HIV-1 *tat* gene was performed by the dideoxynucleotide chain termination method (28).

DNA transfections and growth characteristics of cloned HIV-1 proviruses. To evaluate the biological activity and characteristics of the HIV-1 clones, purified supercoiled plasmid DNA was transfected into PBLs and the H9 and Sup T1 T-cell lines by electroporation as described previously (6). Briefly, coded samples (5 μg) of plasmid DNA were transfected in duplicate into 3-day PHA-prestimulated PBLs (5×10^6) or H9 or Sup T1 cells (3×10^6). After transfection, the cells were washed once. Transfection efficiencies were determined by cotransfecting a chloramphenicol acetyltransferase gene expression vector. Chloramphenicol acetyltransferase enzyme activity was measured as described before (23).

Transfected PBLs were cocultivated with 5×10^6 PHA-stimulated PBLs. The cultures were kept at a cell concentration of 1×10^6/ml (PBLs) or 0.5×10^6/ml (cell lines). Fresh PHA blasts were added to the PBL cultures each week as described before (34). Three times a week, the cultures were examined for syncytium formation by two observers working independently. Every 5 days, supernatant samples were tested in the p24 antigen capture assay. Transmission of virus from PBLs to the H9 and Sup T1 cell lines was attempted by both cocultivation and cell-free transmission (34). Inoculated T-cell line cultures were monitored for syncytium formation and p24 production. The rescue of virus isolates in PBL culture upon transfection in H9 or Sup T1 cells was studied by cocultivation of irradiated (5,000 rads) H9 or Sup T1 cells with PHA blasts on day 3 after transfection. All transfection and transmission experiments were performed at least twice.

RESULTS

Recovery and molecular cloning of HIV-1 isolates. HIV-1 isolates were obtained from an individual whose clinical characteristics have been described before (patient 6 in reference 13). At the time of seroconversion, this patient also had serologic evidence of recent hepatitis B virus and *Treponema pallidum* infection, and the patient developed AIDS within half a year after seroconversion. Clinical data for this patient and the biological phenotypes of the recovered HIV-1 isolates are shown in Table 1. The isolate recovered at the time of seroconversion was of the SI

TABLE 2. Biological phenotypes of HIV-1 isolates used for molecular cloning

Isolate	Biological clone	Syncytium induction in PBLs[a]	Transmission to T-cell lines			
			H9		Sup T1	
			Replication[b]	Syncytium induction	Replication	Syncytium induction
320.2A	1	+	−	−	−	−
	2	−	−	−	+	−
	3	−	−	−	−	−
	4	+	+	+	+	+
320.3		+	+	+	+	+
320.4	3	+	+	+	+	+
320.5		+	+	+	+	+

[a] −, No syncytia observed; +, syncytia induced.
[b] −, Not transmissible; +, transmissible.

phenotype. The HIV-1 isolate obtained 6 weeks after seroconversion was NSI but could be transmitted to the H9 and Sup T1 cell lines by repeated attempts and prolonged coculture (up to 40 days). From 9 weeks after seroconversion onward, a gradual increase in SI capacity and transmissibility to T-cell lines of the HIV-1 isolates was observed and was followed by extremely rapid progression to AIDS 6 months after seroconversion.

In a subsequent experiment from time point 2A (week 9 after seroconversion), direct biological clonal or oligoclonal viral isolates were obtained from patient PBMCs by isolation in microtiter plates. From 94 microtiter well cultures, 37 HIV-1 isolates were obtained, indicating that these isolates were clonal or oligoclonal at most. These clonal isolates exhibited differences in their SI capacity and their ability to replicate in the T-cell lines H9 and Sup T1. From these 37 clones, 4 biological clones with discrete biological phenotypes were selected.

The four biological clones from time point 2A, together with the isolate obtained at time point 3 (week 12), a biological clone obtained by limiting-dilution virus isolation at time point 4 (week 25), and the isolate obtained at time point 5 (week 30), were used as starting material for the molecular cloning. The biological phenotypes of these seven isolates are shown in Table 2. The estimated frequency of HIV-1-containing phages in the libraries generated from infected PBL ranged from 0.25 to 7 per 10^6 screened plaques. The library from H9 cells infected with isolate 320.3 contained 40 HIV-1-positive phages per 10^6 plaques.

Biological properties of infectious molecular HIV-1 clones. A total of 32 plaque-purified molecular HIV-1 clones was obtained from the six λ phage libraries (8 clones from time point 2A, 16 from time point 3, 6 from time point 4, and 2 from time point 5).

The biological activities and phenotypes of 17 of these molecular clones were evaluated by transfection of 3-day PHA-stimulated PBLs (Table 3). Cotransfection experiments with a chloramphenicol acetyltransferase expression vector revealed no significant differences in transfection efficiency between molecular HIV-1 clones (data not shown). Eight of 17 molecular clones (47%) appeared to be infectious, as evidenced by persistent p24 production in PBL culture and infection of fresh PBL cultures with cell-free supernatants of transfected PBL cultures. Analysis of the host DNA flanking sequences demonstrated that these clones all were derived from different proviruses (38).

Of the eight infectious clones, six were SI in PBLs, including clone 320.2A 1.1 and all infectious clones obtained at time points 3 and 5. As for the two remaining infectious clones (both obtained at time point 2A, the point of transition from NSI to SI phenotype), clone 320.2A 1.2 reproducibly exhibited a reduced SI capacity, and clone 320.2A 2.1 was not able to induce syncytia in PBL culture at all (Table 3). This difference in SI capacity was not due to differences in replication rate, since all infectious clones exhibited similar infection kinetics (Fig. 1). When infected cells or virus-containing supernatants were used to infect fresh PBL cultures, the kinetics of infection and the ability to induce syncytia paralleled those seen previously (data not shown).

Transfected PBL cultures at the time of maximal virus production (day 11 to 14 after transfection) were used to attempt virus transmission to the H9 and Sup T1 cell lines. H9 and Sup T1 cells were cocultivated with infected PBLs or infected with cell-free culture supernatants. In agreement

TABLE 3. Infectivity and biological properties of 17 molecular HIV-1 clones

Isolate	Molecular clone	Properties in PBL culture		Transmission to T-cell lines[a]	
		Infectivity[b]	SI capacity[c]	H9	Sup T1
320.2A	1.1	+	+	−	−
	1.2	+	(+)	−	−
	1.3	−			
	1.4	−			
	2.1	+	−	−	+
	3.1	−			
	3.2	−			
	4.1	−			
320.3	1	+	+	+	+
320.3	2	+	+	+	+
320.3	3	+	+	+	−
320.3	4	−			
320.3	5	+	+	+	−
	6	−			
320.4	3.1	−			
	3.2	−			
320.5	1	+	+	+	+

[a] −, Not transmissible; +, readily transmissible.
[b] −, Not infectious; +, infectious.
[c] −, No syncytia observed; (+), reduced SI capacity; +, syncytia induced.

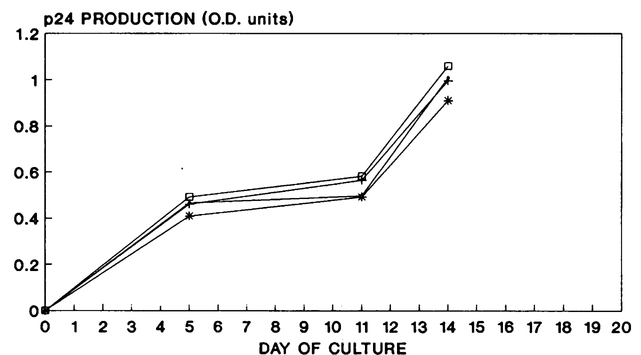

FIG. 1. Equal replication rates of four biologically distinct molecular HIV-1 clones derived from isolates 320.2A and 320.3. PBL cultures were infected with equal amounts (5 μg) of purified plasmid DNA. Results from a representative experiment are shown. Similar results were obtained with the other infectious clones. Symbols: •, 320.2A 1.1; +, 320.2A 1.2; *, 320.2A 2.1; □, 320.3 1.

TABLE 4. Expression of T-cell line-tropic and non-T-cell line-tropic molecular HIV-1 clones upon transfection in T-cell lines

Isolate	Molecular clone	Transmission from PBLs to:		Transfection in T-cell lines				Virus rescue by cocultivation of transfected H9 or Sup T1 cells with PBLs	
				H9		Sup T1			
		H9	Sup T1	Replication[a]	Syncytium induction[b]	Replication[a]	Syncytium induction[b]	Replication[c]	Syncytium induction[d]
320.2A	1.1	–	–	–	–	–	(+)	+	+
	1.2	–	–	–	–	–	(+)	+	(+)
	2.1	–	+	–	–	+	–	+	–
320.3	3	+	+	+	+	–	(+)	+	+
	1	+	+	+	+	+	+	+	+
320.5	1	+	+	+	+	+	+	+	+

[a] –, Only transient p24 production; +, productive infection.
[b] –, No syncytia observed; (+), transient single-cell cytopathic changes; +, syncytia induced.
[c] +, Productive infection.
[d] –, No syncytia observed; (+), reduced SI capacity; +, syncytia induced.

with previous observations of primary HIV-1 isolates, the SI clones from time points 3 and 5 all were able to replicate in the H9 cell line (34). As expected, the NSI clone 320.2A 2.1 did not replicate in the H9 cell line. Like their parental isolate, clone 320.2A 1.1, which induced syncytia indistinguishable from those induced by the clones from time points 3 and 5, and the clone with reduced SI capacity (320.2A 1.2) did not replicate in H9 cells (Table 3).

In studies with primary HIV-1 isolates, we observed that as a rule SI isolates replicate in the Sup T1 cell line as well as some fast NSI variants, particularly when they are derived from patients progressing to AIDS (17a). The transmissibility to Sup T1 cells of two SI clones from time point 3, the SI clone from time point 5, and the NSI clone 320.2A 2.1 is compatible with these findings. However, the SI clones from time point 2A and three of the SI clones from time point 3 did not replicate in Sup T1 cells (Table 3).

T-cell line tropism of HIV-1 clones is not determined at the level of virus expression. To confirm the results of the T-cell line transmission experiments and to determine the stage of the virus replication cycle during which restriction of replication occurs, we proceeded to investigate the effects of transfection directly into the T-cell lines (Table 4). Productive infection of either H9 or Sup T1 cultures was observed only with those clones whose progeny in PBLs was also able to infect these cell lines in the transmission experiments described above. With the other clones, only transient p24 production was observed. These clones also induced some abortive, single-cell cytopathic changes in the Sup T1 cell line.

To find out whether these transfected cultures produced virus able to infect PBLs, we cocultivated PBLs with irradiated Sup T1 or H9 cells harvested 3 days after transfection. In all cases productive infection of the PBL cultures, as evidenced by persistent p24 production, was observed. Moreover, cell-free supernatants from these PBL cultures were able to infect fresh PBL cultures. These experiments indicate that non-T-cell line-tropic HIV-1 clones are properly expressed upon transfection in T-cell lines and that the inability of these variants to productively infect T-cell line cultures is due to a block early in the replication cycle, most probably at the level of entry. The phenotype in PBL culture of the clones passaged through the Sup T1 and H9 cell lines was indistinguishable from the phenotype observed after direct transfection into PBLs (Table 4).

Physical mapping and sequence analysis of the *tat* gene of four HIV-1 clones with divergent biological phenotypes. Among the clones tested for their biological characteristics, the three molecular HIV-1 clones derived from time point 2A (1.1, 1.2, and 2.1) and clone 320.3 1 exhibited the most divergent biological properties. These four clones were analyzed by restriction endonuclease mapping (Fig. 2). The physical maps showed that the four clones were genomically highly related. With the exception of an additional *Nco*I site present in clone 320.2A 1.1, the restriction fragment patterns of 320.2A 1.1 and 320.2A 1.2 appeared to be indistinguishable. Compared with clone 320.2A 1.2, clone 320.2A 2.1 lacked a second *Eco*RV site in the long terminal repeat, an internal *Sst*I site, and three *Hin*dIII sites. However, clone 320.2A 2.1 did contain an additional *Hin*dIII site in the *env* gene. Compared with clone 320.2A 1.2, clone 320.3 1 lacked the first *Eco*RV site in the long terminal repeat and a *Hin*dIII site in the *pol* gene. Clone 320.3 1 contained an additional *Kpn*I site and *Hin*dIII site in the *env* gene. To estimate the overall nucleotide sequence variability, a pairwise comparison of the percentages of restriction site differences between each of the four proviral genomes was performed (26). This approach revealed a maximum estimated nucleotide sequence variability of 3.7% (Table 5). In addition, the nucleotide sequence of the *tat* gene of the four clones was

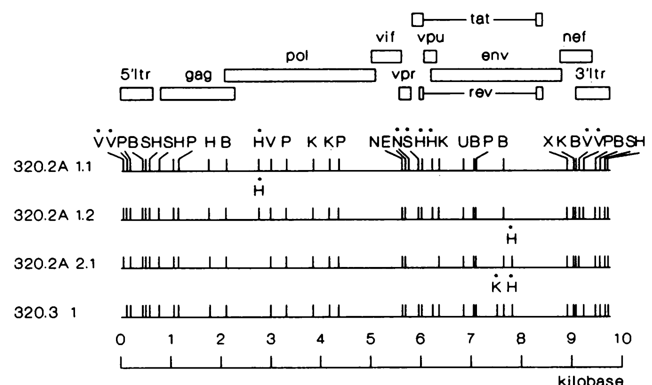

FIG. 2. Physical maps of four biologically distinct molecular HIV-1 clones derived from isolates 320.2A and 320.3. Restriction enzyme cleavage sites: B, *Bgl*II; E, *Eco*RI; V, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; N, *Nco*I; P, *Pvu*II; S, *Sst*I; U, *Stu*I; X, *Xho*I. Asterisks indicate restriction sites not present in all proviruses. *Bam*HI and *Sal*I cleavage sites were not found in any of the four clones. Abbreviation: ltr, long terminal repeat.

TABLE 5. Overall estimated nucleotide sequence differences among four phenotypically divergent HIV-1 molecular clones

Clone	% Sequence difference in comparison with clone:			
	320.2A 1.1	320.2A 1.2	320.2A 2.1	320.3 1
320.2A 1.1				
320.2A 1.2	0.5			
320.2A 2.1	3.7	3.2		
320.3 1	2.5	2.1	3.2	

determined. The *tat* gene nucleotide sequence alignment and the Tat protein amino acid sequence alignment are shown in Fig. 3. The four sequences were different from all other *tat* gene sequences of HIV-1 clones published to date (20, 22). A pairwise comparison of the nucleotide sequences of the *tat* genes of the four biologically distinct HIV-1 variants revealed a maximum nucleotide sequence variation of 3.3% (data not shown), in accordance with the maximum estimated overall nucleotide sequence variation. Moreover, the four Tat proteins were completely homologous in the N-terminal region and the cysteine-rich region (amino acids 1 to 37); the region containing amino acid residues 38 to 48, a possible activating domain; and the nucleolar localization signal and putative nucleic acid-binding region (amino acids 48 to 56), regions of the *tat* gene product critical for its function (14, 16, 24).

DISCUSSION

This article describes the generation and biological characterization of a panel of phenotypically heterogeneous infectious molecular HIV-1 clones derived from a single individual. HIV-1 is notorious for the degree of sequence variability among its different isolates (1, 5). Any attempt to ascribe biological properties to specific genomic regions will be considerably hampered by this extensive background variability. Genetic variation has been shown to be less dramatic among HIV-1 clones derived from the same individual, in particular when the clones are obtained at the same time point (26). Therefore, for our molecular cloning studies we selected an individual from whom sequential isolates with divergent biological phenotypes were previously recovered over a short period.

Previously, we demonstrated that in an individual a transition from NSI to SI isolates may occur, preceding progression to AIDS (35). The individual selected for this study was remarkable in two respects. First, he had a very rapid course of infection, progressing from seroconversion to AIDS within half a year. Second, along with the NSI-to-SI conversion mentioned above, with this individual the reverse phenomenon was observed: at seroconversion an SI isolate was recovered, whereas 6 weeks later an NSI phenotype was observed. Such a suppression of a more virulent HIV-1 phenotype, only temporary in this individual, has recently been described for two infants infected via their mothers (4, 25) and may reflect clearance of SI clones by the still uncompromised anti-HIV-1 immune response (21). The rapid progression of this individual to AIDS may be caused by his apparent inability to persistently suppress the virulent HIV-1 strain with which he had been infected. With this patient, serologic evidence was obtained for hepatitis B virus infection and syphilis at about the time of HIV-1 infection (13), and it is tempting to speculate that these concomitant infections contributed to this patient's rapid clinical deterioration.

To generate HIV-1 clones corresponding as closely as possible to clones occurring in vivo and to avoid selection of clones by PBL bulk culture or transmission to cell lines, we performed molecular cloning of full-length proviral genomes with high-molecular-weight DNA from clonally infected PBL cultures, with the exception of isolate 320.3, which was cloned after transmission to the H9 cell line. Also, the use of oligoclonal isolates with divergent phenotypes increased the chance of obtaining homologous infectious clones with the desired spectrum of biological phenotype. The approximately 10- to 100-fold-higher cloning efficiency from infected H9 cells compared with infected PBLs most probably was due to the higher frequency of infected cells, the higher proviral copy number per cell in the H9 cell line, or both.

As a rule, the phenotypes of the molecular clones matched those of their parental isolates very well. From isolate 320.2A both NSI and SI molecular HIV-1 clones were obtained. The SI clones from this isolate showed no tropism for the H9 cell line, although syncytium induction and H9 cell line tropism are two biological properties almost invariably coupled in field isolates (34, 35). The phenotypic heterogeneity among the molecular clones derived from isolate 320.2A reflected the NSI-to-SI transition occurring at the time point at which this isolate was obtained, which may also account for the presence of SI clones that could not be transmitted to T-cell lines. The finding of SI molecular clones with a host range restricted to PBLs formally demonstrates that SI capacity and H9 tropism are distinct properties.

From isolate 320.3 two SI clones which could be transmitted to the H9 cell line but not to the Sup T1 cell line were obtained. In contrast, the NSI clone 320.2A 2.1 could be transmitted to Sup T1, but not to H9. These findings demonstrate that different requirements exist for H9 and Sup T1 cell line tropism, indicating that there is not a single set of attributes that determines tropism for T-cell lines.

The Tat proteins of four biologically divergent molecular HIV-1 clones did not show amino acid changes in regions which are critical for their function (14, 16, 24). This result is compatible with the finding that in this set of molecular clones, significant differences in replication rate were not observed. This may be related to the fact that the clinical course of HIV-1 infection in this individual lacked a true stable asymptomatic period characterized by the presence of HIV-1 isolates with low replication rates (35).

T-cell line transfection studies confirmed the results obtained by transmission of the clones to the H9 and Sup T1 cell lines. The inability of non-T-cell line-tropic clones to productively infect T-cell line cultures even after direct transfection and the similarity of the biological properties of the clones in PBLs upon passage through T-cell lines demonstrate the phenotypic stability of this panel of HIV-1 clones. Moreover, these experiments provided evidence that T-cell line tropism is not regulated by differential HIV-1 expression but is most likely determined at the level of virus entry, suggesting involvement of the *env* gene.

This study demonstrates that differences in biological phenotype observed among primary HIV-1 isolates can be reduced to single molecular clones. Previous studies have demonstrated the existence of phenotypically heterogeneous HIV-1 clones within one isolate. Fisher et al. observed differences in cytotropism among a panel of hybrid full-length HIV-1 clones which were constructed by insertion of various HIV-1 genome fragments containing the whole *env*

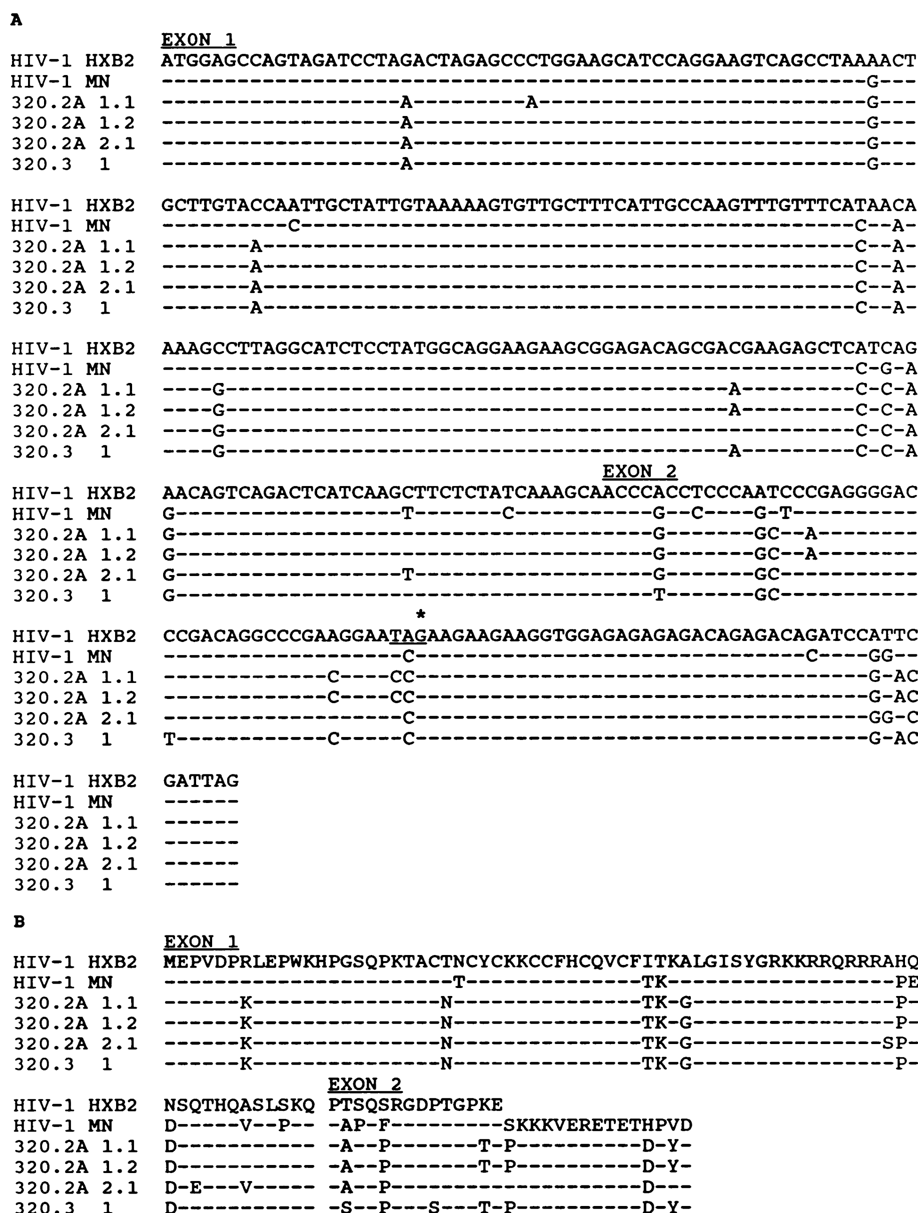

FIG. 3. Alignment of the nucleotide sequences of the *tat* genes (A) and the amino acid sequences of the Tat proteins (B) of the molecular HIV-1 clones 320.2A 1.1, 320.2A 1.2, 320.2A 2.1, and 320.3 1. The nucleotide and amino acid sequences of the molecular clones HIV-1 HXB2 and HIV-1 MN are shown for comparison. The predicted amino acid sequence is presented in the one-letter amino acid code. Dashes indicate nucleotide or amino acid identity. The asterisk indicates a premature stop codon present in HIV-1 HXB2.

gene in a biologically active molecular clone (12). The infectivity of these hybrid HIV-1 clones, however, was not formally proven by virus transmission experiments. Phenotypic variation among infectious molecular HIV-1 clones that were isolated simultaneously from an individual was also reported by Sakai et al. (27). However, in that study, molecular clones were generated from an isolate after passage to and extensive propagation in T-cell lines, thereby excluding the possibility of obtaining clones of non-T-cell line-tropic HIV-1 variants. Similarly, in a study of an attenuated HIV-2 isolate, molecular clones were generated only after passage to the Sup T1 cell line (18).

Earlier studies have indicated that SI capacity is determined primarily by the *env* gene (15, 19, 33). Likewise, the results of our experiments seem to point to the *env* gene as the principal determinant of T-cell line tropism (7, 8). Previously, a number of studies investigating the effects of in vitro mutagenesis of the *env* gene on the biological phenotype of prototype HIV isolates were performed (10, 39). By using this approach, several mutations in the *env* gene that result in changes of biological properties have been identified. From these studies, it also became apparent that similar phenotypic changes may result from mutations at quite different positions in the *env* gene. However, since these mutations have been artificially induced, it is not clear whether these variants also would prevail in vivo in the presence of the host immune response. Therefore, the availability of this panel of highly homologous molecular clones

with stable, distinct biological properties, directly derived from their in vivo environment, provides a useful alternative for the analysis of the in vivo genetic variability responsible for differences in biological phenotype.

ACKNOWLEDGMENTS

This study was supported in part by grant 900-502-104 from the Netherlands Organization for Scientific Research and by grant 28-1079 from the Netherlands Foundation for Preventive Medicine.

We thank Aster Vos for technical assistance.

REFERENCES

1. **Alizon, M., S. Wain-Hobson, L. Montagnier, and P. Sonigo.** 1986. Genetic variability of the AIDS virus. Cell **46:**63–74.
2. **Asjö, B., J. Albert, A. Karlsson, L. Morfeldt-Månson, G. Biberfeld, K. Lidman, and E. M. Fenyo.** 1986. Replicative properties of human immunodeficiency virus from patients with varying severity of HIV infection. Lancet **ii:**660–662.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
4. **Baur, A., N. Schwarz, S. Ellinger, K. Korn, T. Harrer, K. Mang, and G. Jahn.** 1989. Continuous clearance of HIV in a vertically infected child. Lancet **ii:**1045.
5. **Benn, S., R. Rutledge, T. Folks, J. Gold, L. Baker, J. McCormick, P. Feorino, P. Piot, T. Quinn, and M. Martin.** 1985. Genomic heterogeneity of AIDS retroviral isolates from North America and Zaire. Science **230:**1548–1553.
6. **Cann, A. J., Y. Koyanagi, and I. S. Y. Chen.** 1988. High efficiency transfection of primary human lymphocytes and studies of gene expression. Oncogene **3:**123–128.
7. **Cann, A. J., J. A. Zack, A. S. Go, S. J. Arrigo, Y. Koyanagi, P. L. Green, S. Pang, and I. S. Y. Chen.** 1990. Human immunodeficiency virus type 1 T-cell tropism is determined by events prior to provirus formation. J. Virol. **64:**4735–4742.
8. **Cheng-Mayer, C., M. Quiroga, J. W. Tung, D. Dina, and J. A. Levy.** 1990. Viral determinants of human immunodeficiency virus type 1 T-cell or macrophage tropism, cytopathogenicity, and CD4 antigen modulation. J. Virol. **64:**4390–4398.
9. **Cheng-Mayer, C., D. Seto, M. Tateno, and J. A. Levy.** 1988. Biologic features of HIV-1 that correlate with virulence in the host. Science **240:**80–82.
10. **Cordonnier, A., L. Montagnier, and M. Emerman.** 1989. Single amino-acid changes in HIV envelope affect viral tropism and receptor binding. Nature (London) **340:**571–574.
11. **De Wolf, F., J. Goudsmit, D. A. Paul, J. M. A. Lange, C. Hooijkaas, P. T. A. Schellekens, R. A. Coutinho, and J. Van der Noordaa.** 1987. Risk of AIDS related complex and AIDS in homosexual men with persistent HIV antigenaemia. Br. Med. J. **295:**569–572.
12. **Fisher, A. G., B. Ensoli, D. Looney, A. Rose, R. C. Gallo, M. S. Saag, G. M. Shaw, B. H. Hahn, and F. Wong-Staal.** 1988. Biologically diverse molecular variants within a single HIV-1 isolate. Nature (London) **334:**444–447.
13. **Goudsmit, J., F. De Wolf, D. A. Paul, L. G. Epstein, J. M. A. Lange, W. J. A. Krone, H. Speelman, E. C. Wolters, J. Van der Noordaa, J. M. Oleske, H. J. van der Helm, and R. A. Coutinho.** 1986. Expression of human immunodeficiency virus antigen (HIV-Ag) in serum and cerebrospinal fluid during acute and chronic infection. Lancet **ii:**177–180.
14. **Green, M., M. Ishino, and P. M. Loewenstein.** 1989. Mutational analysis of HIV-1 Tat minimal domain peptides: identification of *trans*-dominant mutants that suppress HIV-LTR-driven gene expression. Cell **58:**215–223.
15. **Gruters, R. A., J. J. Neefjes, M. Tersmette, R. E. Y. De Goede, A. Tulp, H. G. Huisman, F. Miedema, and H. L. Ploegh.** 1987. Interference with HIV-induced syncytium formation and viral infectivity by inhibitors of trimming glucosidase. Nature (London) **330:**74–77.
16. **Hauber, J., M. H. Malim, and B. R. Cullen.** 1989. Mutational analysis of the conserved basic domain of human immunodeficiency virus *tat* protein. J. Virol. **63:**1181–1187.
17. **Ho, D. D., T. Moudgil, and M. Alam.** 1989. Quantitation of human immunodeficiency virus type 1 in the blood of infected persons. N. Engl. J. Med. **321:**1621–1625.

17a. **Koot, M., et al.** Unpublished data.

18. **Kumar, P., H. Hui, J. C. Kappes, B. S. Haggarty, J. A. Hoxie, S. K. Arya, G. M. Shaw, and B. H. Hahn.** 1990. Molecular characterization of an attenuated human immunodeficiency virus type 2 isolate. J. Virol. **64:**890–901.
19. **Lifson, J. D., M. B. Feinberg, G. R. Reyes, L. Rabin, B. Banapour, S. Chakrabarti, B. Moss, F. Wong-Staal, K. S. Steiner, and E. G. Engelman.** 1986. Induction of CD4-dependent cell fusion by the HTLV-III/LAV envelope glycoprotein. Nature (London) **323:**725–728.
20. **Meyerhans, A., R. Cheynier, J. Albert, M. Seth, S. Kwok, J. Sninsky, L. Morfeldt-Månson, B. Asjö, and S. Wain-Hobson.** 1989. Temporal fluctuations in HIV quasispecies in vivo are not reflected by sequential HIV isolations. Cell **58:**901–910.
21. **Miedema, F., M. Tersmette, and R. A. W. Van Lier.** 1990. AIDS pathogenesis: a dynamic interaction between HIV and the immune system. Immunol. Today **11:**293–297.
22. **Myers, G., A. B. Rabson, J. A. Berzofsky, T. F. Smith, and F. Wong-Staal.** 1990. Human retroviruses and AIDS. A compilation and analysis of nucleic acid and amino acid sequences. Theoretical Biology and Biophysics, Los Alamos, N.Mex.
23. **Nielsen, D. A.** 1989. A highly sensitive, mixed-phase assay for chloramphenicol acetyltransferase activity in transfected cells. Anal. Biochem. **179:**19–23.
24. **Ruben, S., A. Perkins, R. Purcell, K. Joung, R. Sia, R. Burghoff, W. A. Haseltine, and C. A. Rosen.** 1989. Structural and functional characterization of human immunodeficiency virus *tat* protein. J. Virol. **63:**1–8.
25. **Rubsamen-Waigmann, H., W. R. Willems, U. Bertram, and H. Von Briessen.** 1989. Reversal of HIV-phenotype to fulminant replication on macrophages in perinatal transmission. Lancet **ii:**1155–1156.
26. **Saag, M. S., B. H. Hahn, J. Gibbons, Y. Li, E. S. Parks, W. P. Parks, and G. M. Shaw.** 1988. Extensive variation of human immunodeficiency virus type I in vivo. Nature (London) **334:**440–444.
27. **Sakai, K., S. Dewhurst, X. Ma, and D. J. Volsky.** 1988. Differences in cytopathogenicity and host cell range among infectious molecular clones of human immunodeficiency virus type 1 simultaneously isolated from an individual. J. Virol. **62:**4078–4085.
28. **Sanchez-Pescador, R., and M. S. Urdea.** 1984. Use of purified synthetic deoxynucleotide primers for rapid dideoxynucleotide chain termination sequencing. DNA **3:**339–343.
29. **Schnittman, S. M., M. C. Psallidopoulos, H. C. Lane, L. Thompson, M. Baseler, F. Massari, C. H. Fox, N. P. Salzman, and A. S. Fauci.** 1989. The reservoir for HIV-1 in human peripheral blood is a T cell that maintains expression of CD4. Science **245:**305–308.
30. **Schuitemaker, H., N. A. Kootstra, R. E. Y. de Goede, F. de Wolf, F. Miedema, and M. Tersmette.** 1991. Monocytotropic human immunodeficiency virus type 1 (HIV-1) variants detectable in all stages of HIV-1 infection lack T-cell line tropism and syncytium-inducing ability in primary T-cell culture. J. Virol. **65:**356–363.
31. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. **Simmonds, P., P. Balfe, J. F. Peutherer, C. A. Ludlam, J. O. Bishop, and A. J. Leigh Brown.** 1990. Human immunodeficiency virus-infected individuals contain provirus in small numbers of peripheral mononuclear cells and at low copy numbers. J. Virol. **64:**864–872.
33. **Sodroski, J., W. C. Goh, C. Rosen, K. Campbell, and W. A. Haseltine.** 1986. Role of the HTLV-III/LAV envelope in syncytium formation and cytopathicity. Nature (London) **322:**470–474.
34. **Tersmette, M., R. E. Y. de Goede, B. J. M. 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 detection of syncytium-inducing isolates in patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex. J. Virol. **62**:2026–2032.

35. **Tersmette, M., R. A. Gruters, F. de Wolf, R. E. Y. de Goede, J. M. A. Lange, P. T. A. Schellekens, J. Goudsmit, H. G. Huisman, and F. Miedema.** 1989. Evidence for a role of virulent human immunodeficiency virus (HIV) variants in the pathogenesis of acquired immunodeficiency syndrome: studies on sequential HIV isolates. J. Virol. **63**:2118–2125.
36. **Tersmette, M., J. M. A. Lange, R. E. Y. De Goede, F. De Wolf, J. K. M. Eeftink Schattenkerk, P. T. A. Schellekens, R. A. Coutinho, J. G. Huisman, J. Goudsmit, and F. Miedema.** 1989. Differences in risk for AIDS and AIDS mortality associated with biological properties of HIV variants. Lancet **i**:983–985.
37. **Tersmette, M., I. N. Winkel, M. Groenink, R. A. Gruters, P. Spence, E. Saman, G. van der Groen, F. Miedema, and J. G. Huisman.** 1989. Detection and subtyping of HIV-1 isolates with a panel of characterized monoclonal antibodies to HIV-p24gag. Virology **171**:149–155.
38. **Vink, C., M. Groenink, Y. Elgersma, R. A. M. Fouchier, M. Tersmette, and R. H. A. Plasterk.** 1990. Analysis of the junctions between human immunodeficiency virus type 1 proviral DNA and human DNA. J. Virol. **64**:5626–5627.
39. **Willey, R. L., D. H. Smith, L. A. Lasky, T. S. Theodore, P. L. Earl, B. Moss, D. J. Capon, and M. A. Martin.** 1988. In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity. J. Virol. **62**:139–147.