Relationship between the V3 Loop and the Phenotypes of Human Immunodeficiency Virus Type 1 (HIV-1) Isolates from Children Perinatally Infected with HIV-1

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The third variable region (V3) of the envelope protein of human immunodeficiency virus type 1 (HIV-1) contains group- and type-specific epitopes for neutralizing antibodies and contains determinants involved in viral tropism and syncytium-inducing (SI) activity. We studied the in vivo relationship between V3 sequences and viral phenotypes in 24 perinatally HIV-1-infected children. To avoid in vitro selection of intrapatient minor variants, genetic studies were performed directly on uncultured peripheral blood mononuclear cells (PBMC), and the tropisms of HIV-1 isolates were evaluated by culturing patients' PBMC directly with monocyte-derived macrophages, lymphocytes, and MT-2 cells. According to their phenotypes, we could define five types of primary isolates: (i) non-syncytium-inducing (NSI) macrophagetropic, (ii) NSI macrophage-lymphotropic, (iii) NSI lymphotropic, (iv) SI lympho-T-cell line-tropic, and (v) SI pleiotropic. The SI viral phenotype was correlated with a more advanced status of disease. Genetic analysis of intrapatient molecular variants revealed that no relationship between the degree of intrapatient V3 variability and the pattern of viral tropism existed; moreover, within a single patient, the values for V3 variability between CD4⁺ lymphocytes and CD14⁺ monocytes were similar, thus suggesting that in vivo variability of the monocyto-tropic variants is more extensive than previously appreciated. A comparison between the intrapatient major variants and the phenotype of primary isolates disclosed that a negatively charged amino acid at residue site 25 was associated with an NSI macrophage- and macrophage-lymphotropic viral phenotype. Finally, by comparing the V3 sequences derived from our study population with those of several prototypes, we observed that the majority of isolates circulating in Italy are related to the North American subtype B macrophagetropic isolates.

The third variable region (V3) of the envelope protein of human immunodeficiency virus type 1 (HIV-1) is a disulfide bridge loop of about 35 amino acids and contains determinants that mediate virus interactions with target cells and elicit the host immune response. Antisera to the recombinant Env proteins or to synthetic peptides that include the central 24-amino-acid sequence of the V3 loop, namely, the principal neutralizing domain (26), abrogate infection in chimpanzees (14) and neutralize virus infectivity in vitro (27, 40, 43) by preventing virus entry into target cells after binding to the CD4 receptor (52). Since these neutralizing antibodies are mostly type specific (32-34) and variation within the V3 loop nucleotide sequence has been shown to occur not only between different HIV-1-infected subjects but also according to geographic location (36), the genetic characterization of the V3 domains of the patients' HIV-1 isolates is relevant to a strategy for the development of an immunogen that will neutralize the majority of the circulating indigenous strains.

Furthermore, studies with molecular recombinants indicated that determinants of virus tropism and cytopathic activity lie within the V3 region (3, 6, 25, 49). As disease progression in many HIV-1-infected subjects appears to be accompanied by a shift from non-syncytium-inducing (NSI) monocyto-tropic to syncytium-inducing (SI) T-cell line-tropic isolates (28, 46, 47, 55), these findings confer additional interest on studies aimed at characterizing the V3 sequences of viral strains derived from

patients. Several attempts to correlate the specific patterns of V3 sequence variability with the biological properties of the patients' isolates have been made (7, 18, 30, 38), but most of these studies were conducted on cultured HIV-1 isolates that were derived following in vitro isolation through peripheral blood mononuclear cell (PBMC) culture; as selection of intrapatient viral variants may occur during this process (35), these isolates might misrepresent the in vivo properties of viral strains.

In light of this concern, we addressed the relationship between V3 sequences and viral phenotypes in a series of children with perinatally acquired HIV-1 infection. To avoid any in vitro selection, the patterns of viral tropism and syncytium-inducing capability were determined directly by culturing patient PBMC with indicator target cells, and V3 native sequences were derived directly from uncultured patient cells. In addition, in four available cases, we also analyzed the molecular variants harbored in vivo by CD4⁺ lymphocytes and CD14⁺ monocytes.

Moreover, to address the genetic typing of Italian HIV-1 isolates, we compared the major intrapatient variants with the V3 sequences of several HIV-1 prototypes belonging to the A, B, C, and D HIV-1 subtypes (36). We found that a negatively charged amino acid at position 25 was correlated with a macrophage- or macrophage-lymphotropic NSI phenotype, even if this characteristic was not crucial for in vivo infection of monocytes/macrophages. The majority of the V3 loop sequences observed in our Italian patients are genetically related to subtype B macrophagetropic isolates, and the derived V3 consensus sequence is highly homologous to the reported American consensus sequence.

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MATERIALS AND METHODS

Patients. This study was performed on blood samples obtained from 24 children with perinatally acquired HIV-1 infection who currently attend the Pediatric Department of Padua University. The children were classed as having HIV-related symptoms according to the criteria of the European Collaborative Study (15) and as having AIDS according to the criteria of the Centers for Disease Control (4). None of the children were undergoing antiretroviral therapy at the time of the study. Heparinized blood was collected from each patient, and PBMC were separated by Ficoll-Hypaque gradient centrifugation. After being washed, the cells were resuspended in RPMI medium supplemented with 10% fetal calf serum (FCS) and 10% dimethyl sulfoxide and then cryopreserved until use.

Isolation of CD4⁺ and CD14⁺ cells. Following Ficoll-Hypaque separation, PBMC were double stained with anti-CD14 fluorescein isothiocyanate-conjugated antibody (Mo2; Coulter, Hialeah, Fla.) and with anti-CD4 phycoerythrin-conjugated antibody (CD4; Dakopatts, Glostrup, Denmark). Antibodies were used in saturating conditions according to the manufacturers' instructions. Cells were fixed in cold 1% paraformaldehyde-phosphate-buffered saline (PBS) to prevent virus contamination (11). Cells were sorted with a flow cytometer (Epics-Elite; Coulter); forward-angle and 90°C light scatter gates were used to eliminate debris and select lymphocytes and monocytes in the cell preparation. Sorting criteria were based on fluorescence positivity for CD4 and CD14 antibodies. More than 99% of the recovered cells expressed CD4 or CD14 cell membrane antigen, according to the sorting criteria.

Cells. The CD4⁺ T-cell line MT-2 was maintained at 5×10^5 cells per ml in RPMI medium supplemented with 10% fetal calf serum. Monocyte-derived macrophages (MDM) were obtained from pooled PBMC of HIV-1-seronegative donors, as previously reported (10). Briefly, after Ficoll-Hypaque separation, PBMC were seeded into T25 flasks at a concentration of 5×10^6 cells per ml in endotoxin-free Iscove modified Dulbecco (IMD) medium supplemented with 10% human AB serum (Irvine Scientific, Santa Ana, Calif.) and cultured at 37°C. After 2 days, nonadherent cells were removed and MDM were cultured at a concentration of 10^6 cells per ml in IMD medium supplemented with 10% human AB serum for 3 to 5 days before use in cocultivation experiments. More than 95% of the adherent cells were positive for the CD14 marker, as evaluated by cytofluorimetry with the Mo2 monoclonal antibody.

A T-lymphocyte-enriched cell fraction (peripheral blood lymphocytes [PBL]) was also obtained from the pooled PBMC. Cells were suspended at 5×10^6 cells per ml in RPMI medium supplemented with 10% fetal calf serum and stimulated with phytohemagglutinin (1 μ g/ml) for 48 h; nonadherent cells were harvested, depleted of CD8⁺ lymphocytes by the use of magnetic beads (Dynabeads; Dynal, Oslo, Norway), and resuspended at a concentration of 10^6 /ml in medium supplemented with 10% T-cell growth factor (Cellular Products, Buffalo, N.Y.). More than 95% of the cells were CD4⁺, as evaluated by cytofluorimetry with the CD4 monoclonal antibody.

Viral phenotype analysis. Tropisms and syncytium-inducing capabilities of the viral isolates were evaluated by culturing of patient cells directly with PBL, MDM, and MT-2 target cells. PBL and MDM (10^6 /ml) and MT-2 (2×10^5 /ml) were plated in 24-well plastic tissue culture plates (Costar, Cambridge, Mass.) and cocultured for 28 days with 10^6 patient PBMC. Supernatants were collected twice a week and assayed for p24 antigen (P24 antigen assay; Dupont de Nemours, Wilmington, Del.). A culture was considered positive for HIV-1 when at least two consecutive supernatants were found to be positive (i.e., above the cutoff value of 20 pg/ml). A persistent or increasing p24 value during the culture period was considered a criterion of HIV-1 transmission to target cells. MT-2 cocultures were checked for syncytium formation twice weekly by observation of from 5 to 10 microscopic fields (magnification, $\times 100$) per culture.

PCR and cloning. Following Ficoll-Hypaque separation, 2×10^6 PBMC were washed in PBS and lysed in 500 μ l of a TE buffer (10 mM Tris-HCl [pH 8] and 0.1 mM ethylenediamine tetraacetic acid [EDTA]) containing 0.001% Triton X-100, 0.0001% sodium dodecyl sulfate, and 600 μ g of proteinase K per ml. The V3 region was amplified with the primer pairs V3A-V3D and V3B-V3C, whose sequences were previously reported (50). The primer-binding sites were highly conserved between sequences of geographical variants of HIV-1 (50), and their positions in HIV-1_{IIIIB} (clone HXB2) were as follows (5' to 3'): V3A, 6957 to 6976; V3B, 7009 to 7028; V3C, 7314 to 7331; and V3D, 7361 to 7381 (54). Amplification was accomplished as detailed elsewhere (13) in a 100- μ l reaction mixture containing 25 μ l of lysed cells, 20 nmol of each deoxynucleotide triphosphate (dNTP), 50 pmol each of the outer primers V3A and V3D, and 2 U of *Taq* polymerase. Forty cycles were carried out in a DNA thermal cycler (9600; Perkin-Elmer), each consisting of 40 s at 94°C, 40 s at 49°C, and 1 min at 72°C. Five microliters of amplified DNA was used as template in a nested PCR, with 80 pmol of inner V3B and V3C primers. Forty cycles were run, each of 40 s at 94°C, 40 s at 57°C, and 1 min at 72°C. The expected sizes of the amplified products (424 bp for the PCR with the outer primers and 322 bp for the PCR with the inner primers) were verified by ethidium bromide staining after electrophoresis through agarose gels. Sensitivity of nested PCR was assessed by using 8E51 cells which contain a single proviral DNA copy of HIV-1 per cell (17). These cells were serially diluted with A301 HIV-1-negative cells to obtain standard reference samples containing from 10^4 to 1 HIV-1 copy per 10^5 cells, as previously reported (12). Standard reference samples were lysed and processed

for PCR as described above. After the first round of PCR, an initial input of 100 HIV-1 copies was the smallest amount of starting material that gave a clearly detectable band in an ethidium bromide-stained gel. After hybridization with 5'-end-³²P-labelled V3C oligonucleotide, the detection limit ranged from 1 to 10 HIV-1 copies (see below). In reconstruction experiments with standard reference samples containing a nominal one 8E51 cell, 6 of 10 samples were positive following nested PCR. This result is in agreement with the reported sensitivity of nested PCR to amplify a single HIV-1 copy (51). Positive (reference samples containing 10 or 100 8E51 cells in 10^5 A301 cells) and negative (10^5 A301 cells and lysis buffer) controls were run in all amplification reactions to monitor the sensitivity and specificity of the PCR.

The nested PCR products were purified with the Qiaex gel filtration kit (Qiagen, Dusseldorf, Germany) or the Wizard PCR Preps DNA purification system kit (Promega, Madison, Wis.) according to the manufacturers' protocols. PCR products were cloned with the TA Cloning System kit (Invitrogen, San Diego, Calif.). Four nanograms of DNA was ligated with 50 ng of pCR II vector, and bacterial transformation was accomplished by incubation of competent cells of *Escherichia coli* INV α F' with the ligated vector for 30 s at 42°C in a water bath. Recombinant clones containing the insert were screened on indicator plates containing 50 μ g of ampicillin and 40 μ g of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) per ml. White colonies were selected and cultured, and the DNA from 3-ml cultures was extracted by means of the Wizard Minipreps DNA purification system kit (Promega). The presence of the insert in the plasmid clones was screened by restriction enzyme analysis, which was performed at 37°C for 2 h on 300 to 500 ng of plasmid DNA resuspended in a final volume of 20 μ l containing 10 U of *Eco*RI enzyme (Boehringer, Mannheim, Germany) and 2 μ l of 10 $\times$ buffer (50 mM Tris-HCl, 10 mM MgCl₂, 1 mM dithioerythritol, 100 mM NaCl, pH 7.5). The size of digested DNA was verified by agarose gel electrophoresis and ethidium bromide visualization. A total of 9 to 19 clones from each sample were studied.

Direct PCR products and clones were sequenced by the Sanger dideoxynucleotide method with Sequenase version 2.0 (United States Biochemical, Cleveland, Ohio) as detailed elsewhere (13). For sequencing, the V3B and V3C primers were employed in two different reactions in order to sequence in both forward and reverse directions. Ten clones from two different PCR amplifications of 8E51 cells were sequenced to evaluate the error rate of PCR. No mutations were observed; the estimated error rate ($<1/1,080$ bases sequenced) was consistent with previous observations (35, 41). Sequences from direct PCR products were confirmed by independent analysis of separate amplification reactions performed with the same primer pairs.

Multiplex PCR. A sample of 5×10^4 PBMC and an equal number of the corresponding CD4⁺ and CD14⁺ cells, sorted by the flow cytometer, were lysed with 50 μ l of TE buffer. Although paraformaldehyde-PBS treatment was required for a safe sorting procedure, preliminary experiments disclosed that it slightly decreased PCR efficiency (data not shown); in this set of experiments, therefore, PBMC also underwent this treatment prior to lysis. Amplification was carried out in 100 μ l of reaction mixture containing 50 μ l of cell lysate, 20 nmol of each dNTP, 50 pmol of each V3A-V3D HIV-1-specific primer, 5 pmol of each PCO3-PCO4 primer specific for a 110-bp sequence of the β -globin gene (44), 2 U of *Taq* DNA polymerase, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 1.5 mM MgCl₂. Forty cycles of amplification were carried out, each of 40 s at 94°C, 40 s at 49°C, and 1 min at 72°C. Samples (10 μ l) of the first-round PCR product were electrophoresed on 1% Nusieve and 1% agarose gel, transferred to nylon filters (Hybond-N; Amersham), and then hybridized with the 5'-end-³²P-labelled probes V3C (50) and RSO6 (44), specific for HIV-1 and β -globin sequences, respectively. The sensitivity of PCR was verified by using standard reference samples containing serial dilutions of 8E51 HIV-1-positive cells in 5×10^4 A301 HIV-1-negative cells (12). Reference samples underwent paraformaldehyde-PBS treatment, multiplex amplification, and hybridization exactly as described for patient samples. Nested PCR and cloning of these samples were performed exactly as described above.

Sequence analysis. The sequence analysis programs Microgene (Beckman, Fullerton, Calif.) and Clustal V (24) were used to translate and align V3 sequences. All the sequences obtained were used for calculations of the intrasample and interpatient variability. Calculations were performed with the Kimura's protein distance (KPD) and Dayhoff PAM matrix (DPM) algorithms with the PROTDIST program that is part of the PHYLIP package version 3.5 (16). Intrasample variability was expressed as the mean amino acid distance of all pairwise comparisons between the sequences obtained from a sample; interpatient variability was expressed as the mean amino acid distance of all pairwise comparisons between the major variant sequences obtained from the patient samples. As the large number of pairwise comparisons ($n = 1,600$) made it impractical to present all the values in table form and both algorithms gave similar results, only mean and range values obtained with the KPD method are reported in Results. Comparisons between major intrasample amino acid sequences and HIV-1 prototype sequences were also performed with the KPD and DPM algorithms. Secondary structure prediction for the V3 amino acid sequences was carried out according to the Chou and Fasman method (8).

Nucleotide sequence accession numbers. Patient sequences were deposited in GenBank under accession numbers U09992 to U10015. Direct PBMC nucleotide sequences have GenBank accession numbers U09992 to U09997 and U0999 to

TABLE 1. Clinical statuses of patients and phenotypes of their viral isolates

Patient code	Age (mo)	Clinical status	HIV-1 isolation by PBMC cocultivation with ^a :			Syncytium formation on MT-2 cells
			MDM	PBL	MT-2 cells	
653	36	Asymptomatic	+	(14)	—	—
353	86	Asymptomatic	+	(7)	—	—
565	48	HRS	+	(3)	—	—
675	4	Asymptomatic	+	(17)	+	(10)
566	11	Asymptomatic	+	(14)	+	(7)
706	38	Asymptomatic	+	(7)	+	(10)
671	23	HRS	+	(14)	+	(7)
673	24	HRS	+	(14)	+	(3)
710	68	HRS	+	(10)	+	(7)
623	75	HRS	+	(14)	+	(14)
403	10	AIDS	+	(3)	+	(3)
659	6	Asymptomatic	—	—	+	(10)
462	6	HRS	—	—	+	(7)
563	24	HRS	—	—	+	(7)
354	48	HRS	—	—	+	(3)
666	70	HRS	—	—	+	(10)
661	27	AIDS	—	—	+	(7)
649	32	HRS	—	—	+	(3)
668	3	AIDS	—	—	+	(3)
707	12	AIDS	—	—	+	(3)
556	25	AIDS	—	—	+	(3)
674	25	AIDS	—	—	+	(3)
656	65	AIDS	+	(3)	+	(3)
679	70	AIDS	+	(7)	+	(3)

^a First day of p24 detection is indicated in parentheses.

U10015, and consensus sequence 565 has GenBank accession number U09998; see Fig. 4.

RESULTS

Clinical profiles of the patients and phenotypes of their viral isolates. The characteristics of the 24 infants we studied are summarized in Table 1. In every case, PBMC were cocultured directly with PBL, MDM, and MT-2 target cells to evaluate the tropisms and the syncytium activities of the in vivo-infecting strains (Table 1). By using this approach, five different patterns of tropism were found: three isolates were able to grow only on MDM; eight isolates had tropism for both macrophages and lymphocytes; six isolates had tropism only for lymphocytes, five isolates had tropism for lymphocytes and the MT-2 T-cell line, and two isolates showed tropism for all target cells. These last two types of viral isolates also had SI capability. A correlation of the biological properties of the HIV-1 isolates with the clinical features of the children confirmed the relationship between the viral phenotype and clinical status. None of the six asymptomatic infants harbored T-cell-tropic SI variants, which were instead detected in six of eight infants with AIDS ($P = 0.01$, Fisher's exact test). Conversely, five asymptomatic infants, but only one AIDS patient (number 403, Table 1), had NSI isolates with tropism for MDM ($P = 0.025$, Fisher's exact test). Macrophage tropism and T-cell line tropism appear to exclude each other; indeed only in two AIDS patients (numbers 656 and 679) was HIV-1 isolation achieved in both MDM and MT-2 cells. Interestingly, in two asymptomatic patients (numbers 353 and 653) and in one child with HIV-related symptoms (HRS) (number 565), HIV-1 isolation was successful only in MDM cocultures. This finding stresses the need for culturing patient cells directly with MDM in order to study the in vivo monocytotropic variants, which may instead be lost by

negative pressure during primary isolation in vitro with PBMC used as indicator cells.

Intrasample V3 variability and viral phenotype. One HIV-1 isolate exists in vivo as a mixture of quasispecies molecular variants (35), and it appeared that macrophagetropic isolates are more similar in their sequences than are T-cell-tropic isolates (7). To investigate this aspect in vivo, we studied native V3 sequences from 10 children whose isolates differed in tropism and SI activity. PCR products derived from PBMC were cloned, and 9 to 19 clones from each specimen were analyzed. In addition to sequences from molecular clones, direct sequences from PCR products were also obtained. As shown in Fig. 1, a predominant molecular variant was present in each set of clones. This major variant corresponded to the intrasample-derived consensus sequences as well as to the direct PCR sequence in all cases, except for sample 565, for whom the direct sequencing results were repeatedly ambiguous, therefore precluding comparison. Mean intrasample variability ranged from 0 (patient 707) to 7.3% (patient 565). The complete homogeneity in sequences obtained from patient 707 did not appear to be due to amplification of a limited number of HIV-1 DNA copies and subsequent sequencing of identical copies of a single amplified molecule. Indeed, the first round of PCR of this sample yielded a larger amount of HIV-1 DNA than that detected in sample 565, which presented the highest intrasample variability (Fig. 2B). By comparison of the intensities of the PCR signals with those obtained from reference samples, viral burdens of about 100 and of <50 HIV-1 copies per 5×10^4 cells could be estimated in PBMC of patients 707 and 565, respectively (Fig. 2B and C). Differences between intrasample major and minor variants were due both to randomly occurring substitutions, which were generally observed in only one sequence of each set of clones, and to substitutions at a specific site, mainly at positions 9, 13, and 29 of the V3 loop, which were instead detected in more than one sequence in one or more sets of clones. Interestingly, the substitutions at residues 13 and 29 occurred mainly with histidine and aspartic acid, which were the most common amino acids at these positions, respectively. Sample 565 showed the highest intrapatient variation; the majority of its clones, as well as the derived consensus sequences, contained 37 amino acids; indeed, two insertions were observed in the carboxy-terminal region of the V3 loop. Minor variants with one or two deletions were also observed; this variability in nucleic acid and sequence length could explain the failure to derive the V3 domain by direct sequencing.

No relationship was found between the values of V3 intrapatient variability, viral isolate tropism, and disease status. Two patients (656 and 707) with a highly homogeneous viral population had both AIDS and a polytropic SI isolate, as did patients 674 and 403, who conversely showed mean intrasample variabilities of 3.7 and 2.0%, respectively. Moreover, patients 661 and 565, who had NSI isolates with tropisms for only lymphocytes and only macrophages, respectively, showed higher mean intrasample variabilities (3.1 and 7.3%, respectively) than patients 656 and 707.

V3 loop sequences of viral variants in lymphocytes and monocytes in vivo. To investigate the relationship between V3 loop sequences and in vivo tropisms more thoroughly, CD4⁺ lymphocytes and CD14⁺ monocytes were obtained from the PBMC of four children from whom isolates with different phenotypes had been derived. These cells were separated by flow cytometry according to their positivity for CD4 or CD14 surface marker (Fig. 2A). PBMC and the two cell fractions were coamplified with the V3A-V3D HIV-1 primers and β -globin primers. After first-round PCR, 10- μ l samples of the

FIG. 2. Viral burden in cell subsets in vivo. Lymphocytes and monocytes were sorted by cytofluorimetry from PBMC of four patients and analyzed by PCR. (A) Fluorescence criteria used for sorting $CD4^+$ and $CD14^+$ cells in the representative sample 675. (Left) Forward-angle and 90° light scatter gates were used to eliminate debris and select lymphocytes and monocytes (bitmap a, left panel). (Right) Two-parameter analysis of CD4 and CD14 log fluorescence gated on bitmap a. Sorting criteria for lymphocytes and monocytes were defined as $CD4^+$ (bitmap b) and $CD14^+$ (bitmap c). (B) A sample of 5×10^4 total PBMC and equal numbers of corresponding $CD14^+$ and $CD4^+$ cell fractions were coamplified with V3A-V3D HIV-1-specific primers and PCO3-PCO4 β -globin-specific primers. Samples (10μ l) of PCR products were electrophoresed and hybridized with V3C and RSO6 probes specific for HIV-1- and β -globin-amplified products, respectively. (C) PCR results of standard reference samples. Positive samples (8E51 cells, which contain a single proviral DNA copy per cell, serially diluted in 5×10^4 A301 HIV-1-negative cells), an HIV-1-negative sample (5×10^4 A301 cells), and an HIV-1- and β -globin-negative control (lysis buffer) were processed exactly as described for panel B.

acid sequences of the molecular clones are shown in Fig. 3, aligned with the consensus sequences derived from the corresponding PBMC molecular clones. In each patient, the PBMC consensus sequence was also detected in the corresponding cell fractions and represented the major variant in every sample except in the $CD4^+$ fraction of sample 565. Indeed, the major variant observed in this last cell sample constituted only a minor variant in the corresponding set of PBMC molecular clones; this finding is compatible with the low HIV-1 burden observed in the $CD4^+$ cells of this child.

Interestingly, in the same patient, the values for V3 variability in the $CD14^+$ cells did not differ significantly from those in $CD4^+$ cells, and V3 variability values in both cell fractions were similar to those observed in the corresponding total PBMC. For example, patient 707 showed a highly homogeneous population in both $CD4^+$ and $CD14^+$ cells, in agreement with data found in the PBMC; patient 565, whose PBMC showed the highest intrasample variability, also had high intrasample variabilities in both $CD4^+$ (mean, 3.9%; range, 0 to 12%) and $CD14^+$ (mean, 5.4%; range, 0 to 12%) cells.

No. 565 PBMC consensus		C T R P N I N K S K R L S I G P G R A F V A A R Q I T I T G D I R R A H C																			
CD4+ clones	6/11 •	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2/11 •	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2/11 •	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1/11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	(3.9; 0-12.0)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CD14+ clones	5/10 •	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2/10 •	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1/10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1/10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1/10 •	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(5.4; 0-12.0)		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
No. 666 PBMC consensus		C T R P N N N T R K G I H M G P G K T F Y V • • T G E I V G D I R Q A H C																			
CD4+ clones	5/9 •	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2/9 •	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1/9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1/9 •	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(5.4; 0-15.8)		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
No. 675 PBMC consensus		C T R P N N N T R K S I P I G P G R A F Y T • • T G E I I G N I R Q A H C																			
CD4+ clones	5/10 •	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3/10 •	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2/10 •	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(2.4; 0-5.9)		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CD14+ clones	4/10 •	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2/10 •	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2/10 •	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2/10 •	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(3.1; 0-5.9)		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
No. 707 PBMC consensus		C T R P N N N T R K S I H I G P G R A F Y T • • T G D I I G D I R Q A H C																			
CD4+ clones	7/7 •	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	(0; 0-0)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CD14+ clones	4/5 •	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1/5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(1.1; 0-2.9)		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

FIG. 3. V3 variability in CD4⁺ and CD14⁺ cells. Amplified products obtained by nested amplification of CD4⁺ and CD14⁺ cells were cloned, and 5 to 10 clones for each sample were sequenced. In each set of clones, the deduced amino acid sequences were aligned with the corresponding PBMC consensus sequence. • indicates sequences that were also found in the clone set obtained from the corresponding PBMC (see Fig. 1). The means and ranges of intrasample variability are reported in parentheses. —, amino acid homology; *, amino acid deletion.

It is important to note that in each patient the pattern of viral variants detected in PBMC was highly consistent with that observed in the corresponding fractionated cell subsets. As shown in Fig. 2 and 3, 35 of 36 variants that were detected in at least two clones and 7 of 17 variants detected in a single clone were recovered in both clone sets derived from independent amplifications of unfractionated PBMC and corresponding lymphocyte and monocyte cell fractions. Thus, of the total of 117 sequences derived from PBMC and cell subsets of these four patients, only 12 (10.2%), corresponding to 16 base mutations in the 12,405 bases sequenced (0.13%), could not be confirmed in the independent analyses of unfractionated and corresponding cell fractions. It is possible that these minor underrepresented variants were generated *in vitro* by *Taq* enzyme copying error and/or lost during the different amplification and cloning procedures. The overall consistency of findings achieved in the independent analyses of unfractionated and fractionated cell populations strongly indicated that the patterns of intrapatient variants reflected the *in vivo* naturally occurring viral populations and argued against the possibility that they were generated *in vitro* by a selective amplification of some variants or by PCR and cloning artifacts.

Correlation between the V3 sequence of the intrasample major variant and the phenotype of the viral isolate. The above findings showed that, regardless of the biological behavior of the viral isolate, the intrasample major variants corresponded to those derived by direct sequencing of PBMC. Despite the possibility that minor variants might be selected *in vitro* in different target cells, it was likely that the intrasample major variant mainly determined the host immune response to the V3 loop and the prevailing phenotype of the patient isolate. Therefore, in order to derive the most representative intrapatient viral variant, we determined the V3 domain of the isolates from the other 14 children directly, by sequencing the PCR product without any previous cloning. This approach gave satisfactory results in every case and thus confirmed a relatively high intrasample homogeneity. Figure 4 shows the deduced amino acid sequences of these specimens aligned with the amino acid sequences derived from the major intrasample variants of the previously described cases. The V3 consensus sequence derived from all of these specimens was consistent with the North American consensus sequence (31); at position 25, glutamic acid was present in 9 of the 24 specimens, and a conservative substitution from glutamic to aspartic acid occurred in 9 other samples. The mean interpatient variability was 19.8%, with ranges of 2.9 (between patients 353 and 403) to 57.8% (between patients 565 and 666), and as expected, was considerably higher than the intrapatient variability. The distribution of the amino acid divergence per site, calculated as the number of sequences different from the consensus sequence, was not uniform along the V3 domain. Both the amino- and carboxy-terminal regions were highly conserved among the isolates, and the central GPGR tetrapeptide motif, a feature of the North American isolates (31), was conserved in 17 of the 24 isolates. The most variable positions were located at the flanking region of the V3 tip; besides the conservative

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	1	10	20	30	35
AM	C	R	S	I	H
CONSENSUS	C ₁₀₀ T ₉₆	R ₉₁ K ₈₃ S ₈₃ I ₉₆ H ₇₀ I ₈₃ G ₉₁ P ₉₆ G ₁₀₀ R ₈₃ A ₈₃ F ₉₆ Y ₉₂ T ₄₆ *	*	T ₉₁ G ₈₃ X	I ₉₆ I ₇₉ G ₁₀₀ D ₈₃ I ₁₀₀ R ₁₀₀ Q ₈₇ A ₁₀₀ H ₁₀₀ C ₁₀₀
Pt No. 653	-	-	-	-	-
Pt No. 353	-	-	-	-	-
Pt No. 565	-	S	-	-	R
Pt No. 675	-	-	-	-	-
Pt No. 566	-	R	-	-	-
Pt No. 706	-	-	Q	-	-
Pt No. 671	-	-	Q	-	-
Pt No. 673	-	G	-	-	-
Pt No. 710	-	-	-	-	-
Pt No. 623	-	-	-	-	-
Pt No. 403	-	-	-	-	-
Pt No. 659	-	R	-	-	-
Pt No. 462	-	-	K	-	K
Pt No. 563	-	G	-	-	H
Pt No. 354	-	-	-	-	-
Pt No. 666	-	G	-	-	-
Pt No. 661	-	-	-	-	-
Pt No. 649	-	R	-	-	-
Pt No. 668	-	-	-	-	-
Pt No. 707	-	-	-	-	-
Pt No. 556	-	-	-	-	-
Pt No. 674	-	S	-	-	-
Pt No. 656	-	-	-	-	-
Pt No. 679	-	-	-	-	-

FIG. 4. Major intrasample variants in 24 patients (Pt). The direct PBMC sequence is reported for every patient except 565, for whom the consensus sequence deduced from 15 clones is shown. For details, see the text. The V3 amino acid sequences are aligned with the derived consensus sequences. —, amino acid homology; *, amino acid deletion; □, basic amino acid; ▤, acidic amino acid.

TABLE 2. Amino acid distances in the V3 region between inpatient major variants and viral prototypes

Patient isolate no.	Amino acid distance (%) between inpatient major variant and indicated HIV-1 prototype by indicated algorithm ^a									
	BAL1		MN		U455		NOF		ELI	
	KPD	DPM	KPD	DPM	KPD	DPM	KPD	DPM	KPD	DPM
653	9.12	8.29	27.32	26.02	28.29	26.80	27.32	26.66	67.03	71.12
353	0.00	0.00	19.52	20.11	28.29	26.10	31.52	29.22	59.43	62.71
565	40.35	38.85	40.35	40.69	65.41	64.68	57.85	56.17	102.74	92.43
675	5.95	5.52	23.32	23.24	32.67	29.70	35.96	32.66	59.43	63.80
566	12.43	11.56	35.96	35.98	42.23	38.04	35.96	33.55	67.03	67.99
706	15.89	14.11	35.96	33.53	24.13	22.49	15.89	15.91	59.43	62.04
671	0.00	0.00	19.52	20.11	28.29	26.10	31.52	29.22	59.43	62.71
673	9.12	8.47	23.32	25.14	32.67	30.02	31.52	31.02	67.03	69.36
710	2.92	0.80	19.52	19.13	28.29	26.39	31.52	29.61	59.43	62.39
623	5.95	5.61	23.32	23.13	24.13	23.17	35.96	33.99	52.44	57.42
403	2.92	2.69	23.32	23.30	28.29	27.75	31.52	30.84	59.43	65.04
659	20.18	19.39	37.31	36.69	43.94	42.97	37.31	37.87	70.81	76.46
462	5.95	5.55	27.32	27.53	37.31	33.75	40.65	36.55	75.37	76.73
563	19.52	18.68	31.52	34.72	42.23	40.85	40.65	41.68	84.57	82.15
354	5.95	5.49	23.32	22.57	24.13	22.83	27.32	26.16	59.43	63.18
666	19.52	17.99	40.65	42.88	37.31	34.97	31.52	32.06	75.37	78.86
661	9.12	8.72	23.32	22.98	32.67	29.60	35.96	32.66	59.43	65.17
649	9.41	8.72	24.13	23.13	33.91	31.14	32.67	30.36	62.61	63.19
668	15.89	14.49	35.96	34.33	37.31	34.94	35.96	33.95	75.37	73.65
707	5.95	5.67	27.32	28.20	32.67	30.30	31.52	29.87	75.37	79.08
556	15.89	17.99	31.52	32.64	37.31	36.88	35.96	35.47	75.37	81.79
674	5.95	5.67	23.32	24.13	24.13	23.66	31.52	29.71	52.44	55.38
656	24.13	23.33	37.31	36.10	49.44	45.97	32.67	34.90	62.61	60.53
679	15.89	14.48	35.96	35.71	37.31	35.48	50.92	45.48	67.03	67.66
Mean	11.58	10.92	28.76	28.83	34.68	32.69	34.63	33.15	67.03	69.20

^a Percentages of amino acid distance between inpatient major variants and viral prototypes of B (BAL1 and MN), A (U455), C (NOF), and D (ELI) HIV-1 subtypes were calculated with KPD and DPM algorithms.

substitution at site 25 from glutamic acid to aspartic acid, the polar uncharged threonine at site 22 was frequently replaced by hydrophobic nonpolar alanine (37.5%).

A higher net charge of the V3 domain and nonconservative basic amino acid substitutions at residue sites 11, 24, 25, and 32 have been directly implicated in the shift from macrophage- to T-cell line-tropism (38). Moreover, a correlation between a basic amino acid at positions 11 and/or 25 and transitions from NSI to SI phenotype has been reported (18). A comparison between the V3 sequences of the major variants in vivo and those of our patients' isolates disclosed that 10 of 11 macrophagetropic and macrophage-lympho-dualtropic NSI isolates had aspartic acid or glutamic acid at residue site 25 and a lower net positive charge at the V3 domain (mean $\pm$ standard deviation, 3.10 ± 0.56), while four of the seven lympho-T-cell-tropic and pleiotropic SI isolates (556, 656, 674, and 679) showed a basic or uncharged amino acid at this position ($P = 0.05$, Fisher's exact test) and a higher, but not statistically significant, net charge (4.0 ± 0.8).

Comparison of intrasample major variants with HIV-1 prototypes. V3 sequences of intrasample major variants were compared with those of HIV-1 prototypes classed, according to their Env and Gag sequences, as A, B, C, and D subtypes of HIV-1 (36). The pairwise comparisons performed according to the KPD algorithm disclosed that our isolates are more closely related to the BAL1 and MN B subtype strains (Table 2). It is notable that two V3 sequences (353 and 671 in Table 2) were identical to the BAL1 strain, while no identity was found with MN and the other isolates compared. In addition, the mean variability in our samples and in BAL1 was consistently lower (11.58%) than that observed with MN itself (28.77%) and with strains U455 (subtype A) (34.68%), NOF (subtype C)

(34.63%), and ELI (subtype D) (67.03%). As shown in Table 2, sequence analysis according to the DPM algorithm gave very similar results.

The secondary structure predictions of the V3 sequences were derived according to the Chou and Fasman algorithm (8) and are given in Fig. 5. Of 24 sequences, 23 were predicted to have a structure with a β -turn at the amino-terminal region, a β -strand on the top of the V3 loop, and an α -helix at the carboxy-terminal region. Interestingly, the V3 sequence from patient 565, which showed a major divergence from all HIV-1 subtypes, was predicted also to have a different secondary structure with an α -helix tendency on the central portion of the V3 loop.

DISCUSSION

In this study, we addressed the relationship between the in vivo patterns of V3 sequences and the biological phenotypes of HIV-1 viruses harbored in children with perinatally acquired HIV-1 infection.

By culturing patient PBMC directly with target cells, we established the existence of a pattern more complex than that found when viruses derived by in vitro isolation through PBMC coculture are studied. Indeed, besides their lymphotropism, a prerequisite for in vitro isolation on PBMC culture, primary isolates are usually divided into two types according to their growth patterns on macrophages and T-cell lines (5, 46–49). Our approach enabled us to define five patterns of viral tropism; accordingly, patient isolates could be macrophage-tropic, macrophage-lympho-dualtropic, lymphotropic, lympho-T-cell line-dualtropic, and pleiotropic with tropism for all three target cell types. Rather than an absolute preclusion of growth

MN	P N D																			
	C	T	R	P	N	Y	N	K	R	K	R	I	H	I	G	P	G	R	A	F
	t	t	t	t		t	α	α	α	β	β				t			α	β	β
BAL	S S D																			
	C	T	R	P	N	N	T	R	K	S	I	H	I	G	P	G	R	A	F	Y
	t	t	t	t		t					β			t			α	β	β	β
Pt No. 653	t	t	t	t		t					β			t			β	β	β	t
Pt No. 353	t	t	t	t		t					β			t			α	β	β	t
Pt No. 565	t	t		β		t		α	α		β			t			α	α	α	α
Pt No. 675	t	t	t	t		t					β			t			α	β	β	t
Pt No. 566	t	t	t	t		t	t		β		β			t	t		β	β	β	β
Pt No. 706	t	t	t	t		t					β	β		t			β	β	β	t
Pt No. 671	t	t	t	t		t					β			t			α	β	β	t
Pt No. 673	t	t	t	t		t	t				β			t			α	β	β	β
Pt No. 710	t	t	t	t		t					β			t			α	β	β	t
Pt No. 623	t	t	t	t		t					β			t			α	β	β	t
Pt No. 403	t	t	t	t		t					β	β		t			α	β	β	t
Pt No. 659	t	t	t	t		t	t		β	β	β	β	t	t		α	α	β	β	t
Pt No. 462	t	t	t	t		t					β	β		t			α	β	β	t
Pt No. 563	t	t	t	t		t	t				β			t			α	β	β	β
Pt No. 354	t	t	t	t		t					β	β	β	t			α	β	β	t
Pt No. 666	t	t	t	t		t	t				β	t		t			β	β	β	β
Pt No. 661	t	t	t	t		t					β			t			α	β	β	t
Pt No. 649	t	t	t	t		t	t		β		β			t			α	β	β	t
Pt No. 668	t	t	t	t		t		α	β	t				t			α	β	β	t
Pt No. 707	t	t	t	t		t					β	β	β	t			α	β	β	β
Pt No. 556	t	t	t	t		t	t				β			t			α	β	β	α
Pt No. 674	t	t	t	t		t					β			t			α	β	β	β
Pt No. 656	t	t	t	t		t	t		β		β		β	t	t		β	β	β	β
Pt No. 679	t	t	t	t		t					β			t			α	β	β	t

FIG. 5. Secondary structure predictions for the V3 loops of inpatient major variants. Predictions were derived according to the Chou and Fasman algorithm (8). The principal neutralizing domain (PND) and the serotype-specific domain are indicated for the reference strains MN (T-cell-tropic prototype of B subtype) and BAL1 (macrophage-tropic prototype of B subtype). α, α-helix; β, β-strand; t, β-turn; Pt, patient.

of macrophage- and lymphomonotropic isolates in vitro in lymphocytes and macrophages, respectively, it may be possible that isolates exhibit a gradient of tropism, as suggested by Gendelman and coworkers (21); at low viral doses, a given isolate may be able to infect only its more permissive cell type. In this setting, the findings that the isolate from patient 565 was able to grow only on MDM and the isolate from patient 707 lacked macrophage tropism, despite the presence in vivo of low virus levels in CD4⁺ lymphocytes and CD14⁺ monocytes, respectively, support this idea. The capability of some patient strains to grow better in monocyte/macrophage cells than in lymphocytes also stresses the need to use MDM as target cells in the primary isolation of in vivo-occurring strains, particularly for studies of HIV-1 biological phenotype.

Genetic analysis disclosed that the means of V3 intrapatient variabilities ranged from 0 to 7.3%. Evidence that viral variants and variability values in PBMC were highly consistent with those observed in the independent analyses from corresponding lymphocyte and monocyte cell fractions strongly indicates that they reflect the in vivo viral population patterns rather than being generated in vitro as amplification and cloning procedure artifacts. It has been reported that HIV-1-infected newborns harbor a highly homogeneous viral population, and it has been suggested that a single genotype is transmitted from mother to child (45, 57). The heterogeneity we found in our samples might be explained by the more advanced ages of our infants and thus by the longer period between transmission and time of sampling. No significant relationship was found between the extents of V3 variability in vivo and the tropisms of the patient isolates; patient 656 clearly documented that a wide tropism can be sustained by a highly homogeneous population of molecular variants; conversely, V3 heterogeneity can be associated with a restricted tropism, as observed in patient 565.

In addition, we found that the values for V3 variability of the molecular variants harbored in vivo by CD14⁺ and CD4⁺ cells were quite similar within a patient. Although observed in a small number of cases, this finding indicates that the in vivo variability of the monocytotropic variants is more extensive than previously appreciated. As both the rate of HIV-1 replication and the immune response to replicating virus contribute to the emergence of viral heterogeneity (37, 39), it is tempting to advance the proposition that the variants harbored in monocytes in vivo undergo, at least in some cases, the same replication patterns and immune pressures as those harbored in the lymphocytes. The recent evidence that HIV-1 infection lacks a true latent phase (42), even during the clinically asymptomatic period when monocyte/macrophage-tropic isolates are more often detected, and the fact that neonatal monocytes/macrophages also appear to have an increased susceptibility to HIV-1 infection compared with adult monocytes/macrophages (53) might further support this suggestion.

A recent comparative analysis of a large set of V3 loop sequences derived from primary or culture-adapted isolates disclosed that an acidic amino acid or alanine predominated at position 25 among the macrophage-tropic isolates while a basic or uncharged amino acid at this site was associated with nonconservative basic amino acid substitutions mainly at positions 11, 24, and 32 and that both these features correlated with the T-cell line tropisms of the viral isolates (38). Moreover, in a series of primary isolates, it was demonstrated that NSI isolates had fewer positively charged V3 amino acid sequences than did SI isolates and had an uncharged or negative amino acid at residues 11 and/or 25 (18). Furthermore, the presence of an acidic amino acid or alanine at position 25 and of tyrosine at position 21 have been associated with macrophage tropism (56). The majority of sequences we

found in vivo, according to the Milich et al. classification (38), are of the macrophagetropic type. An examination of the V3 sequences of intrapatient major variants and the biological phenotypes of our patient isolates disclosed that a negatively charged amino acid at position 25 correlated with a macrophagetropic or macrophage-lympho-dualtropic NSI viral phenotype. It is important to mention that genetic analysis of V3 sequences harbored in CD14⁺ cells in vivo in patient 565 demonstrated that this feature is not a prerequisite for in vivo infection of monocytes; however, in this case the entire V3 loop sequence showed a particular pattern in both sequence and secondary structure. In addition, in our samples the substitutions occurring at position 25 were mainly replacements by an uncharged amino acid; moreover, no significant correlation between other predicted positions and viral phenotypes was found. A striking difference between other investigations and our study was that we determined the phenotypes of the patients' isolates directly without previous viral isolation in primary PBMC cells; since in vitro culture contributes to selection of viral variants, it may also have influenced the biological phenotypes of the primary isolates. Overall, while our in vivo data confirm and support the relevance of the amino acid at position 25 of the V3 loop sequence in determining the viral phenotype as reported in other studies (9, 18, 38, 49, 56), they also demonstrate that the V3 region alone cannot constitute a genetic marker for the viral phenotype. Other epitopes very likely contribute to the HIV-1 in vivo tropism and cytopathic activity. In this regard, the V1 and V2 regions of the gp120 envelope protein have been demonstrated to be involved in tropism and/or SI capability of molecular recombinant viruses (1, 2, 19, 23) and of primary isolates (23). Changes in the amino-terminal region of the gp41 protein also have been reported to affect tropism (20) and cytopathic properties of molecular recombinant viruses (29). Studies of naturally occurring viruses in vivo and examination of viral variants emerging from primary coculture of patient cells with different target cells are needed in order to provide a better understanding of the genetic determinants and viral phenotypes of Env.

Genetic analysis of the V3 loop disclosed a mean interpatient variability of 19.8% (range, 2.9 to 57.8%). A comparison between the sequences of our patients and several prototypes of HIV-1 subtypes revealed that homology was highest with the viruses belonging to the B subtype of HIV-1, which includes the prevailing North American and European strains (31). Within this subgroup, homology was consistently higher with the BAL1 macrophagetropic prototype than with the MN T-cell-tropic prototype, thus further confirming that in vivo most of the sequences are compatible with a monocyto-tropic or monocyte-lympho-dualtropic phenotype. The lowest degree of homology was found with the African isolate ELI, which belongs to the D subtype of HIV-1. The isolate most distantly related to the prototypes compared was found in patient 565. It is worth mentioning that all our patients live in northern Italy and that all samples were derived from children who acquired HIV-1 infection from drug-addicted mothers. We advance the suggestion that the source of viral contamination in the mother of child 565 differed from that of the major circulating Italian strains.

In a previous study, we defined a 12-amino-acid domain within the V3 loop, namely, the specific-serotype domain (SSD), which correlated with the presence of high-affinity/high-avidity antibodies to PND (13) and overlapped with the type-specific neutralization determinant described by Goudsmit et al. (22). The linear sequence and the secondary structure of the SSD, predicted according to the Chou and Fasman algo-

rithm, are very closely related to those of the BAL1 B subtype strain in 11 of 24 cases. Since neutralizing antibodies can be directed against linear and conformational epitopes of the V3 loop, our finding that most of the Italian specimens we examined were genetically and conformationally related to the North American B macrophagetropic subtype will aid in the design of appropriate vaccines.

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