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Macrophage-Tropic Human Immunodeficiency Virus Isolates from Different Patients Exhibit Unusual V3 Envelope Sequence Homogeneity in Comparison with T-Cell-Tropic Isolates: Definition of Critical Amino Acids Involved in Cell Tropism

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Previous experiments indicate that the V3 hypervariable region of the human immunodeficiency virus (HIV) envelope protein influences cell tropism of infection; however, so far no consistent V3 sequence can account for macrophage or T-cell tropism. In these experiments, we studied infectious recombinant HIV clones constructed by using V3 region sequences of HIV isolates from 16 patients to search for sequences associated with cell tropism. Remarkable homology was seen among V3 sequences from macrophage-tropic clones from different patients, and a consensus V3 region sequence for patient-derived macrophage-tropic viruses was identified. In contrast, V3 sequences of T-cell-tropic clones from different patients were highly heterogeneous, and the results suggested that sequence diversity leading to T-cell tropism might be generated independently in each patient. Site-specific mutations identified amino acids at several positions on each side of the GPGR motif at the tip of the V3 loop as important determinants of tropism for T cells and macrophages. However, a wide variety of mutant V3 sequences induced macrophage tropism, as detected in vitro. Therefore, the homogeneity of macrophage-tropic patient isolates appeared to be the result of selection based on a biological advantage in vivo.

Cell specificity of infection by human immunodeficiency virus (HIV) appears to vary among virus strains and may be an important determinant of AIDS pathogenesis. Viruses isolated during the early asymptomatic phase after seroconversion may be less pathogenic than viruses isolated during later disease stages associated with severe AIDS. These HIV isolates appear to differ in ability to induce formation of large multinucleated syncytial cells in cultures of peripheral blood mononuclear cells (PBMC) and some T-cell leukemia lines (2–4, 29). Syncytium induction in vitro leads to cell death and may be involved in depletion of CD4-positive lymphocytes associated with immunosuppression in vivo. In contrast, HIV isolates lacking syncytium-forming ability for T lymphocytes still replicate in PBMC cultures but in addition show high replication levels in macrophage cultures (10, 11, 23). Such macrophage-tropic HIV clones also replicate well in brain microglial cultures (16) and therefore are likely to be involved in the AIDS dementia syndrome.

Several groups have shown that sequences including the V3 envelope region can influence tropism for macrophages or T-cell leukemia lines (5, 15, 21, 25, 30, 31). We recently derived a line of CD4-positive HeLa cells (clone 1022) which was susceptible to infection by most, but not all, HIV isolates made directly from human patients (7). Using infectious recombinant HIV constructs made from HIV molecular clones differing in tropism for macrophages, T-cell leukemia lines, and 1022 cells, we found that infectivity for T-cell leukemia cells correlated with infectivity for 1022 cells but was usually inversely correlated with infectivity for macrophages (5). In these studies, a 120-bp region in the V3 region of the envelope protein was able to influence tropism of infectivity. However, since the number of clones studied was small and the V3 region is known to have extensive amino acid sequence variability, it was unclear exactly what amino acids account for differences in cell tropism.

In this study, we compared two groups of HIV isolates which differed in infectivity for 1022 cells and macrophages in order to examine which combinations of amino acid sequences in the V3 region could be associated with different patterns of cell tropism. The results indicated that V3 sequences of macrophage-tropic isolates from different patients were highly homogeneous, in contrast to the diversity seen in V3 sequences of the T-cell-tropic isolates. The findings suggested that diversity associated with T-cell tropism was generated independently in each individual, whereas significant selective pressures appeared to maintain the homogeneity of macrophage-tropic strains in different patients. Because mutagenesis studies showed that sequences associated with macrophage tropism in vitro were not limited to only those sequences found in macrophage-tropic patient isolates, factors other than macrophage tropism itself appeared to select for the homogeneous sequences associated with this phenotype in vivo. We propose here that one such selective factor might be efficiency of natural virus transmissibility between individuals.

MATERIALS AND METHODS

Cell culture and HIV isolation and preparation. HIV was isolated from PBMC of HIV-seropositive individuals by cocultivation with normal donor phytohemagglutinin (PHA)-stimulated PBMC as described previously (7). Plasmid DNA from molecular clones NL4-3 (1) and JR-CSF (18) was used

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to transfect cells to produce infectious HIV as previously described (5). HIV stocks of the macrophage-tropic isolate BA-L (10), HIV stocks of the macrophage-tropic molecular clone JR-FL (18), and patient viruses were amplified by one cell-free passage on fresh donor PBMC. Isolates 13539, Q13, BA-L, K8, and 6859 were biologically cloned by two endpoint dilutions on PBMC.

PCR amplification and cloning of HIV sequences. DNA for the polymerase chain reaction (PCR) was prepared from cells of the second PBMC culture or later PBMC cultures in the case of biologically cloned isolates noted above. Because we were unable to amplify sufficient DNA for cloning by using the previously described *Stu*, *Mlu*, and *Nhe* oligonucleotides (5) directly on DNA from HIV-infected PBMC cultures, a nested PCR was used. The first PCR was carried out with oligonucleotides 6575 (*Dra*; 5′-GTA AAA TTA ACC CCA CTC TGT GT-3′) and 7330C (*Mst*; 5′-CGT GCG GCC CTC AAC AAT TAA AAC TGT GC-3′) for 30 cycles at 94°C for 1 min, 50°C for 30 s, and 72°C for 2 min in a reaction volume of 25 to 50 μl containing 0.1 μM oligonucleotides and 2.5 to 5 μl of DNA extract plus buffers and *Taq* polymerase as previously described (5). This reaction produced a strong 0.8-kb band from DNA of all HIV-infected cultures. A second PCR reaction was carried out by using 0.1 to 0.5 μl of DNA from the first PCR with upper-strand oligonucleotide primer 6813 (*Stu*) or 7097 (*Mlu*) and the lower-strand primer 7262C (*Nhe*) (5), which amplified a region of DNA within the previously amplified DNA. After the second PCR reaction, DNA was digested with appropriate restriction enzymes (*Stu*I or *Mlu*I and *Nhe*I) and ligated into the p9-6 or p4-14 vector. p9-6 was prepared by replacing the HIV envelope gene in pNL4-3-10-17 from *Stu*I (6822) to *Nhe*I (7250) with an oligonucleotide linker containing *Stu*I, *Mlu*I, *XbaI*, *Sac*II, and *Nhe*I sites (5). p4-14 was prepared by reinserting the NL4-3 envelope sequence from *Stu*I to *Mlu*I into p9-6, using DNA amplified from pNL4-3 with oligonucleotides 6813 (*Stu*) and 7129C (*Mlu*) (5). Clones were selected by hybridization with a synthetic oligonucleotide from this region. Plasmid DNA containing recombinant HIV constructs was used to transfect 1022 cells by the calcium phosphate method or PHA-stimulated PBMC by using hypotonic shock and DEAE-dextran as previously described (5). At 24 h after transfection, 1022 cells were cocultivated for 24 h with fresh 3-day PHA-stimulated PBMC; 24 h later, suspended PBMC were removed to new culture wells containing fresh PHA-stimulated PBMC and cultured as usual to allow virus to spread. Virus stocks were made from the PBMC culture supernatant fluid taken just prior to the initial peak of reverse transcriptase release.

Site-specific mutagenesis of V3 region sequences. Mutant HIV recombinant clones were constructed by substituting synthetic DNA fragments between the *Mlu*I and *Nhe*I sites of the NL4-3 vector p4-14 described above. DNA fragments of 120 bp with protruding ends compatible with *Mlu*I and *Nhe*I sites were constructed by synthesizing three upper-strand oligonucleotides and three lower-strand oligonucleotides capable of hybridizing to form a double-stranded DNA of desired sequence. Oligonucleotides were phosphorylated at the 5′ ends with polynucleotide kinase, annealed, and then ligated into the p4-14 HIV vector. The DNA sequence for each *Mlu*I-to-*Nhe*I insert was verified by dideoxy sequencing of the plasmid clones obtained.

Cell tropism studies. Infectivity assays in clone 1022 CD4-positive HeLa cells, blood-derived macrophages, and CEM cells were performed as described previously (5, 6) except that replication in macrophages and CEM cells was measured by p24 antigen capture assay of culture supernatant fluid, using a commercial kit (Cellular Products, Inc., Buffalo, N.Y.) or similar assay with an anti-HIV p24 monoclonal antibody (clone 183-H12-5C) selected in our laboratory. Foci of HIV antigen-positive macrophages were also analyzed by the indirect immunoperoxidase method. Macrophage cultures were fixed for 10 min with 3% H_2O_2 in methanol to inactivate endogenous peroxidases prior to immunostaining with mouse monoclonal antibody reactive with HIV p24 and detection with peroxidase-conjugated anti-mouse immunoglobulin (6).

RESULTS

Cell tropism of HIV patient isolates. Initially, 1022 cells were used to detect T-cell-tropic viruses because most fresh HIV isolates from patients infect 1022 cells much better than T-cell leukemia lines (7). From over 100 patient HIV isolates made in PBMC in our laboratory, we found only seven isolates which failed to infect 1022 cells, and we obtained three additional 1022-negative isolates (BA-L, JR-CSF, and JR-FL) from other laboratories (10, 18) (Table 1). We compared these 10 1022-negative isolates with five patient isolates and one molecular clone (NL4-3) which had high infectivity for 1022 cells (Table 1). The clinical status of the patients in both groups ranged from asymptomatic to AIDS with opportunistic infection, dementia, or Kaposi's sarcoma. All isolates had high infectivity for PBMC, and all isolates showed some infectivity for macrophages. However, in macrophage cultures, 1022-negative isolates usually had higher p24 levels (range, 350 to 250,000 pg/ml) than did 1022-positive isolates (range, 17 to 1,790 pg/ml) (Table 1). Similar conclusions were reached when macrophage infectivity was analyzed by the number of foci of macrophages staining positive for HIV antigens per culture (Table 1). When tested for infectivity on CEM T-cell leukemia cells, of all the 1022-positive and -negative isolates in Table 1, only NL4-3 showed rapid high replication in CEM cells detectable by p24 assay (>1,000 pg/ml).

Cell tropism of recombinant molecular clones derived from patient isolates. In contrast to our previous experiments using recombinant molecular clones (5), the studies described above showed that all 1022-positive isolates had some infectivity for macrophages. This discrepancy might be due to the presence of heterogeneity in the uncloned patient HIV isolates. To circumvent this problem, we constructed recombinant infectious molecular clones by using PCR to amplify a 0.43-kb *Stu*I-to-*Nhe*I or 0.12-kb *Mlu*I-to-*Nhe*I HIV DNA fragment from the V3 region of the HIV envelope genes in our isolates. These molecules were ligated into the NL4-3 infectious molecular clone, and the plasmid DNA was analyzed by sequencing. Virus stocks were produced in PBMC cultures and tested for infectivity in various cell types. In all clones, the donor of the V3 region appeared to be able to influence the cell tropism observed. All of the clones derived from 1022-positive isolates retained infectivity for 1022 cells and also showed good replication in the CEM line of T-cell leukemia cells, whereas clones derived from 1022-negative isolates showed significantly lower replication in CEM cells and were negative in 1022 cells (Fig. 1). High infectivity for 1022 and CEM cells correlated very well, despite the fact that many of the original virus isolates failed to replicate efficiently in CEM cells (Table 1). Thus, sequences of the CEM-adapted NL4-3 vector from outside the V3 region insert area appeared to contribute to the increased replication of 1022-positive clones in CEM cells. In macro-

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TABLE 1. Comparison of infectivity of HIV isolates on PBMC, CEM cells, HeLa 1022 cells, and macrophages[a]

Isolate	Diagnosis[b]	CD4 count[c]	Infectivity				
			HeLa 1022 cells[d]	CEM cells[e]	Macrophages		PBMC[f]
					Foci[g]	p24[h]	
1022 cell infective							
NL4-3[i]			2,000	20,000	0	17	10,000
9539	AIDS-KS	748	30,000	102	0	350	100,000
14558	Asymptomatic	434	6,000	<7	1	48	1,000
14114	Asymptomatic	314	6,000	85	22	1,560	10,000
13231	AIDS	174	50,000	200	150	1,790	100,000
13539	AIDS	225	20,000	340	15	740	10,000
1022 cell noninfective							
JR-CSF	AIDS-KS-dementia	NA[j]	<3	78	95	761	1,000
JR-FL	AIDS-KS-dementia	NA	<3	NT	>400	33,000	10,000
K3	Asymptomatic	1,760	<3	<7	18	1,640	100,000
K7	AIDS	113	<3	184	>400	101,000	10,000
Q13	AIDS	36	<3	<7	>400	250,000	100,000
391	NA	NA	<3	<7	>400	17,000	10,000
Q15	AIDS	135	<3	44	>400	7,000	100,000
BA-L	NA	NA	<3	230	>400	≥200,000	10,000
K8	AIDS	136	<3	96	>400	13,000	10,000
6859	Asymptomatic	1,040	<3	<7	32	350	100,000

[a] All isolates studied were propagated in PHA-stimulated PBMC. The JR-CSF isolate was obtained from cerebrospinal fluid, and the JR-FL isolate was obtained from frontal lobe brain tissue of the same patient (18). All other isolates were made from peripheral blood. Infectivity data are means of two to four experiments.
[b] Symptomatic AIDS patients had AIDS with dementia, opportunistic infection, or Kaposi's sarcoma (AIDS-KS). Asymptomatic seropositive individuals are also noted.
[c] Determined on the day of bleeding for virus isolation.
[d] Expressed as focus-forming units per 0.2 ml (6).
[e] Expressed as supernatant p24 (picograms per milliliter) at 14 days after infection. NT, not tested.
[f] Expressed as 50% tissue culture infective doses per 0.2 ml.
[g] Expressed as number of foci of HIV-positive macrophages per TC48 culture well on days 17 to 20 after infection with 0.2 ml of undiluted virus stock.
[h] Expressed as supernatant p24 (picograms per milliliter) at 9 or 10 days after infection of cultures as previously described (5).
[i] Since NL4-3 is a hybrid molecular clone derived from two separate patients (1), no diagnosis or CD4 count is shown.
[j] NA, not available.

phages, most clones showed some detectable infectivity; however, compared with 1022-positive clones, 1022-negative clones had significantly higher levels of macrophage infectivity detectable by both p24 assay and HIV-positive macrophage foci. Only five 1022-negative clones had p24 values less than 100 pg/ml, and all of these clones also had low 50% tissue culture infective dose values in PBMC (Fig. 1). Repeated attempts to raise higher-titered stocks from these clones were not successful.

For all isolates for which 0.12-kb *Mlu*I-to-*Nhe*I inserts were derived secondarily from 0.43-kb *Stu*I-to-*Nhe*I clones, the patterns of tropism observed were identical in both clones. Therefore, sequence data are shown only for the *Mlu*I-*Nhe*I region plus seven amino acids upstream of the *Mlu*I site, starting at the beginning of the V3 region (Fig. 1). The GPGR sequence often seen at the tip of the V3 loop, and known to influence cell fusion (9), was seen in most of the clones in both groups and therefore did not determine cell tropism. There was no single amino acid at any position in the sequence which correlated precisely with infectivity for either 1022 cells or macrophages. In fact, most of the 1022-positive clones in Fig. 1 had the amino acid substitutions previously associated with macrophage tropism (31) (H, Y, and D or E at positions marked by arrows in Fig. 1 and 2) but still had very low infectivity for macrophages.

Although the molecular clones derived from different patients were remarkably similar, typically having only one to five amino acid differences over the 54 amino acids compared in Fig. 1, the 1022-positive and -negative clones from different patients had a striking difference in amino acid sequence heterogeneity in the V3 region. The 1022-positive macrophage-negative clones derived from different patients showed broad sequence diversity at many positions (Fig. 2). In contrast, the 1022-negative macrophage-positive clones from different patients were quite homologous to each other, showing 78 to 96% homology to the JR-CSF-derived recombinant clone 10-26 (Fig. 1) and having a good consensus sequence (Fig. 2). These results suggested that strong selective pressure appeared to maintain the relative homogeneity of the 1022-negative macrophage-positive viruses, whereas considerably more diversity was associated with 1022-positive T-cell-tropic viruses.

Cell tropism of clones made by mutagenesis of the V3 region. To test the effects of the various amino acid sequence differences seen between 1022-positive and -negative clones, we made mutations in the region between *Mlu*I and *Nhe*I sites. Because of the unique nature of the V3 sequence of the NL4-3 clone, which has two unusual amino acids inserted just upstream of the GPGR motif, we did not attempt to mutate this sequence. Instead, the sequence of the 1022-negative JR-CSF clone 10-26 was altered to introduce amino acids found in 1022-positive clones 22-16 and 21-14 from patients 13539 and 14114 in Fig. 1. For all mutant clones, high infectivity for 1022 cells correlated with a high replication level in CEM cells (data not shown). The critical regions for 1022 and CEM cell tropism were located on both sides of the GPGR sequence (Fig. 3). In the case of clone 123, which was identical to patient-derived clone 22-16, this region consisted of seven amino acids substituted between positions 13 and 30; in clone 146, which was identical to patient-derived clone 21-14, this region consisted of five amino acids substituted between positions 11 and 20 (Fig. 3).

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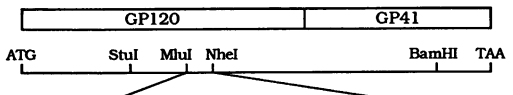

ISOLATE	CLONE	V3 REGION	PERCENT HOMOLOGY NL4-3	PERCENT HOMOLOGY JR-CSF	INFECTIVITY HELA 1022	INFECTIVITY CEM (P24)	INFECTIVITY MAC (FOCI)	INFECTIVITY MAC (P24)	INFECTIVITY PBMC
NL4-3	9-21 (MN)	CTRPNNN TRK.SIRIQR GPGRAFVTIG KI.GNMRQAH CNISRAKWNA TLKQIAS	100	76	2,900	190,000	nt	28	1,000
9539	53-14 (SN)	----Y-K IRRNR-H-.. -----.Y-TE Q-T-DIGR-- -TLNKI--VN --R----	46	62	50	510,000	0	< 7	100
14558	55-17 (SN)	----HST --R.R-H-.. ------Y-TK D-Q-T----Y -T-NKEN-GK --E----	54	62	1,600	220,000	1	30	10,000
	55-18 (SN)	----HST --R.R-H-.. ------Y-TK D-Q-T----Y -T-NKEN-GK A-E----	52	60	2,600	160,000	2	18	10,000
14114	56-22 (SN)	------Q EQIRH-H-.. ------Y.TR NRI-DI-R-- --L---S--N --N----	54	70	1,300	330,000	0	31	10,000
	56-23 (SN)	------T KKIRR-H-.. ------Y.TR DI.-DI-R-- --L---S--N --N----	54	70	6,300	230,000	3	36	10,000
	57-26 (SN)	------Q EKIRH-H-.. ------Y.TR NRI-DI-R-- --L---S--N --N----	54	70	1,400	790,000	2	23	1,000
	57-27 (SN)	------Q EKIRH-H-.. ------Y.TR NGI-DI-R-- --L---S--N --N----	54	70	1,200	520,000	1	25	1,000
13231	21-14 (MN)	------- ---.R-TM.. ----VY Y-T- E-I-DI-K-- -T-NK----E -------	63	76	405	200,000	0	< 7	100
13539	40-16 (SN)	------Y --R.--P-.. ------R-T. Q-V--LK--Q -SL--TQ--N --H----	62	68	4,500	270,000	0	< 7	1,000
	40-15 (SN)	------Y --R.--S-.. ------R-T. Q-V--LK--- --L--TQ--N --H----	65	72	2,400	480,000	2	60	10,000
	22-16 (MN)	------- --R.--S-.. ------R-T. Q-V--L---- --L--TQ--N --H----	69	74	32	52,000	0	< 7	100
JR-CSF	10-26 (MN)	----S-- ---.--H-.. ------Y-T- E-I-DI---- ------Q--N -------	77	100	< 3	250	0	1,180	1,000
JR-FL	58-17 (MN)	------- ---.--H-.. ------Y-T- E-I-DI-K-- ------Q--N -------	77	96	< 3	525	3	50	100
	58-34 (MN)	------- ---.--H-.. ------Y-T- E-I-DI---- ------Q--N -------	79	98	< 3	570	22	86	10
K3	23-20 (MN)	------- ---.--H-.. A-----YAT- E-I-DI---- ---------N -------	75	92	< 3	1,690	150	3,040	1,000
	31-13 (SN)	---A--- ---.--H-.. A-----YAT- E-I-DI---- ---------N -------	73	90	< 3	120	nt	430	1,000
K7	32-4 (SN)	------- --R.--H-.. A-----YAT- E-I--I---- ---------N --I----	73	86	< 3	< 50	3	44	100
	24-22 (MN)	------- --R.--H-.. A-----YAT- E-I--I---- ---------N --I----	73	86	< 3	1,250	80	710	310
Q13	33-42 (MN)	------- ---.--H-.. ------YAT- E-I--I---- --L-ITN--N --R----	69	84	< 3	1,630	TN	4,400	100
391C	36-15 (SN)	------- --R.--N-.. ------Y-T- E-I-DI---- ---------N -------	77	92	< 3	260	34	340	1,000
	26-29 (MN)	------- --R.--N-.. ------Y-T- E-I--I---- ---------N -------	79	90	< 3	740	23	650	100
Q15	35-13 (SN)	----S-- --R.--PM.. ---G-IYAT- Q-I-DI---- ----KTQ-EN -V-----	58	78	< 3	100	nt	69	10
	25-25 (MN)	------- --R.--PM.. ---G-IYAT- Q-I-DI---- ----KAQ-EN -------	62	82	< 3	1,150	150	5,370	1,000
BAL	49-4 (SN)	------- ---.--N-.. ------Y-T- E-I-DI---- --L------D --N----	75	88	< 3	180	9	132	1,000
	49-5 (SN)	------- ---.--H-.. ------Y-T- E-I-DI---- --L------D --N----	75	90	< 3	< 50	90	1,030	100
	52-6 (MN)	------- ---.--N-.. ------Y-T- E-I-DI---- --L------D --N----	75	86	< 3	< 50	12	92	10
K8	51-9 (SN)	------- ---.--H-.. -----WYAT- D-I-DI---- --L---D--N --E----	69	86	< 3	6,800	TN	12,000	10,000
	51-10 (SN)	------- ---.--H-.. -----WYAT- D-I-DI---- --L---D--N --E----	69	86	< 3	5,100	80	1,300	1,000
	28-32 (MN)	------- ---.--P-.. ------Y-T- D-I--I---- --L-IT---N -------	75	86	< 3	690	11	295	1,000
6859	29-34 (MN)	------- ---.--H-.. -----WYAT- D-I-DI---- --L---D--N --E----	69	86	< 3	3,500	46	2,070	1,000

FIG. 1. V3 region amino acid sequence and cell tropism of recombinant molecular clones constructed from patient HIV isolates. DNA from recombinant plasmids was sequenced by using dideoxy sequencing of plasmid DNA. The amino acid translation of sequences of the V3 region and 16 amino acids downstream up to the *Nhe*I site are shown. The prototype sequence of NL4-3, clone 9-21, containing an introduced *Mlu*I site at nucleotide 7121 (5), is shown on the top line. In subsequent sequences, dashes represent amino acids identical to those in the clone 9-21 sequence. Substituted amino acids are indicated with single-letter codes, and deletions are marked with dots. Clones shown contained inserts from *Stu*I to *Nhe*I (SN) or *Mlu*I to *Nhe*I (MN), as indicated. Arrows mark three amino acid positions previously associated with macrophage tropism (31). Boxed sequences show homologous regions found in all 1022-negative clones and in one 1022-positive clone (21-14). Infectivity studies were done as described in Materials and Methods and the footnotes to Table 1. MAC, macrophages; nt, not tested; TN, too numerous to count.

Despite the similar locations these critical motifs, clones 123 and 146 differed significantly from each other in sequence. Thus, the combinations of particular groups of amino acids in each clone appeared to be important. For example, the VY sequence at positions 19 and 20 in clone 146 was associated with 1022 cell tropism only in combination with the RTM motif at positions 11, 13, and 14. All clones with infectivity for 1022 cells also induced foci of fused multinucleated 1022 cells detectable 48 h after transfection of these cells with viral DNA. Amino acid changes in clones 233, 133, and 231 abolished this fusion ability for 1022 cells. However, these clones lacked the ability to replicate in 1022 cells, macrophages, or PBMC, so these mutations also appeared to inhibit some aspect of replication common to all cell types tested.

In many instances, mutations which abolished infectivity for 1022 cells resulted in a switch to macrophage tropism. The same mutant clones shown in Fig. 3 are presented in Fig. 4, which demonstrates the localization of JR-CSF sequences associated with macrophage tropism. As few as two or three amino acids from JR-CSF at any one of several locations on either side of the GPGR motif were sufficient to induce macrophage tropism (Fig. 4, clones 126, 147, and 148). One clone, 129, had properties of combined tropism for macrophages, 1022 cells, and CEM cells. Thus, in clone 129, the JR-CSF sequence from positions 32 to 44 appeared to induce macrophage tropism even in the presence of the upstream substitutions from positions 11 to 20, which was associated with 1022 and CEM cell tropism.

In all cases, macrophage infectivity could be detected both by p24 assay and by appearance of macrophages expressing HIV antigens seen by immunoperoxidase staining. In most cultures, significant syncytium formation resulting from fusion of macrophages could be observed (Fig. 5). Thus, although these viruses did not induce syncytia in PBMC, CEM cells, or 1022 cells, they were fully capable of extensive syncytium induction in macrophages. This was similar to the fusion observed in central nervous system (CNS) tissues of patients with AIDS dementia (14, 17, 27) and may be an important in vivo pathogenic consequence of infection by HIV strains with macrophage tropism.

DISCUSSION

Our previous studies using transfection of infectious DNA as well as pseudotyped virus particles containing HIV genomes plus envelope proteins from other retroviruses such as amphotropic murine leukemia virus or human T-cell

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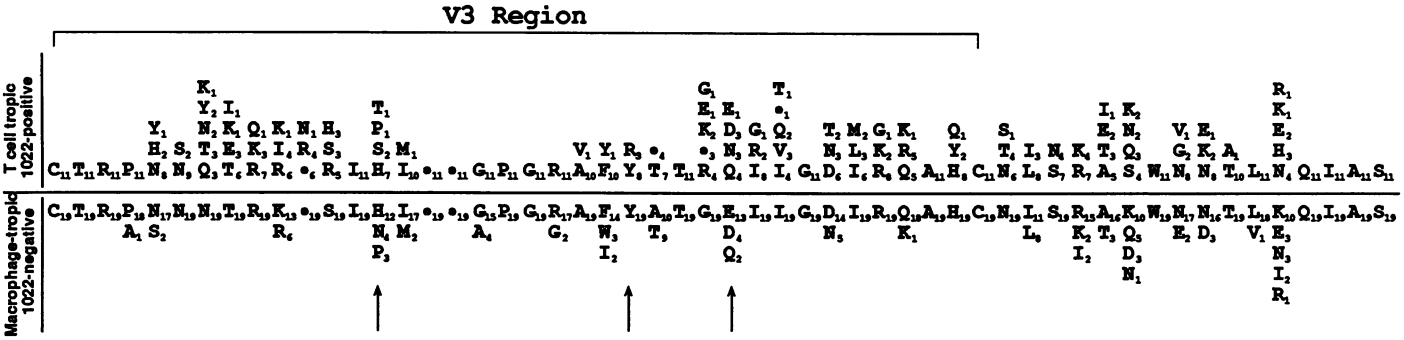

FIG. 2. Frequency of amino acids at each position within and near the V3 region of the HIV envelope protein in recombinant HIV clones depicted in Fig. 1. Subscript numbers represent the number of clones having a particular amino acid at each position. The consensus sequences derived by these comparisons are unavoidably skewed by the fact that different numbers of clones from the various patients were analyzed and clones from the same individual were more closely related than were clones from different individuals. Arrows mark amino acids at three positions previously associated with macrophage tropism (31). In sequences shown, most of the amino acids at these positions in macrophagetropic clones were also found in some T-cell-tropic clones. Thus, these amino acids alone did not appear to account for macrophage tropism.

leukemia virus type I or II indicated that infectivity for 1022 cells was regulated by variation in the virus-cell fusion steps of the viral entry process (5). Others have reached similar conclusions concerning infectivity of T-cell leukemia lines (21, 25). The actual biochemical mechanism which allows 1022 cells, CEM cells, and macrophages to distinguish different HIV envelope proteins is still unclear. It is possible that this mechanism involves activation of a fusion activity in the viral envelope protein by a cellular protease (8, 13). Such a protease might be specific for the envelope amino acid sequence of a particular HIV strain; alternatively, the envelope proteins of individual HIV strains might show a cellular specificity in their fusion and entry functions.

These studies using mutant clones localized sequences on both sides adjacent to the GPGR motif at the tip of the V3 loop as being required for T-cell tropism (Fig. 3). In contrast, macrophage tropism could be induced by sequences in any of four different locations within the V3 region (Fig. 4). In the dual-tropic clone 129, the cell-type-specific functions of these sequences appeared not to interfere with each other. It may be that such dual-tropic viruses are important in the in vivo natural evolution of new mutants differing in pathogenesis. Macrophage-tropic HIV strains have been isolated from CNS tissue, and some clones have been shown to replicate in CNS microglial cells, which are related to macrophages (16). However, all patients infected with macrophage-tropic isolates do not have clinical evidence for CNS HIV infection. It is possible that some of these patients

NL4-3 RECOMBINANT CLONES	V3 REGION (MluI 10 … 20 … 30 … 40 … NheI)	INFECTIVITY: HELA 1022	MACROPHAGES FOCI	MACROPHAGES P24	PBMC
JR-CSF(10-26)	CTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHCNISRAQWNNTLKQIAS	< 3	0	1,180	1,000
123(22-16)	R S R •Q V NL L T H	5,800	0	< 7	10,000
125	R S R •Q V NL L T	4,700	1	45	10,000
134	S R •Q V NL L T H	140	0	< 7	100
230	R S R •Q V NL	9,300	1	< 20	10,000
233	R S R	< 3	NT	NT	<1
133	R R •Q V NL L T H	< 3	NT	NT	<1
231	R S •Q V NL L T H	< 3	NT	NT	<1
126	R •Q V NL L T H	< 3	100	1,365	100
145	R S	< 3	67	975	100
144	R	< 3	150	1,410	1,000
143	S	< 3	32	480	1,000
146(21-14))	R TM VY K T NK K E	405	0	10	100
232	R TM VY K	9,000	2	30	1,000
129	R TM VY	230	19	515	100
149	K T NK K E	< 3	7	67	10
147	R TM K T NK K E	< 3	125	1,048	100
148	VY K T NK K E	< 3	43	181	10
135	R TM	< 3	62	1,362	1,000
136	VY	< 3	91	1,175	1,000

FIG. 3. Influence of mutations within and near the V3 region on HIV cell tropism. The prototype clone shows the *Mlu*I-to-*Nhe*I region substituted from clone JR-CSF as in clone 10-26 of Fig. 1. In subsequent clones, only the amino acids derived from 1022-positive, CEM-positive clones are shown. Amino acid substitutions associated with high infectivity in 1022 and CEM cells are boxed. Infectivity assays were done as described in Materials and Methods and the footnotes to Table 1. Clones 233, 133, and 231 did not produce infectious virus stocks after transfection of either 1022 cells or PBMC. These clones did induce HIV antigen expression 48 h after transfection of 1022 cells, but no 1022 cell fusion was observed. NT, not tested

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NL4-3 RECOMBINANT CLONES	V3 REGION (MluI … NheI)	INFECTIVITY HELA 1022	MACROPHAGES FOCI	MACROPHAGES P24	PBMC
123	CTRPNNNTRRSISIGPGRAFRTT•QIVGNLRQAHCNLSRTQWNNTLHQIAS (1, 10, 20, 30, 40, 50)	5,800	0	< 7	10,000
125	K	4,700	1	45	10,000
134	K	140	0	< 7	100
230	I A K	9,300	1	< 20	10,000
233	GE I DI I A K	< 3	NT	NT	< 1
133	H	< 3	NT	NT	< 1
231	Y	< 3	NT	NT	< 1
126	K H	< 3	100	1,365	1 00
145	Y GE I DI I A K	< 3	67	975	100
144	H Y GE I DI I A K	< 3	150	1,410	1,000
143	K Y GE I DI I A K	< 3	32	480	1,000
JRCSF (10-26)	K H Y GE I DI I A K	< 3	0	1,180	1,000
146	CTRPNNNTRKRITMGPGRVYYTTGEIIGDIRKAHCTINKAKWENTLKQIAS	405	0	10	100
232	N SR Q N	9,000	2	30	1,000
129	Q N SR Q N	230	19	515	100
149	S HI AF	< 3	7	67	10
147	AF	< 3	125	1,048	100
148	S HI	< 3	43	181	10
135	AF Q N SR Q N	< 3	62	1,362	1,000
136	S HI Q N SR Q N	< 3	91	1,175	1,000
JRCSF (10-26)	S HI AF Q N SR Q N	< 3	0	1,180	1,000

FIG. 4. Influence of mutations within and near the V3 region on HIV cell tropism. Clones are the same as those shown in Fig. 3. However, the prototype clones are the 1022-positive, CEM-positive clones 123 (top) and 146 (bottom) from patients 13539 (clone 22-16) and 13231 (clone 21-14). In subsequent clones, only the amino acids substituted from macrophage-tropic clone JR-CSF are shown, and the regions associated with macrophage tropism are boxed. Clones 233, 133, and 231 did not produce infectious virus stocks after transfection of either 1022 cells or PBMC. These clones did induce HIV antigen expression 48 h after transfection of 1022 cells, but no 1022 cell fusion was observed. NT, not tested.

have asymptomatic CNS infections. Alternatively, further mutations of viral sequences may be required to create viruses with high potential for CNS infectivity and pathogenicity in vivo.

These results confirm the importance of the V3 envelope region in influencing cell tropism of clones derived from 15 different patient HIV isolates. However, the most striking finding of this study was the difference in V3 region sequence homogeneity seen between macrophage-tropic compared with T-cell-tropic clones detected in 1022 or CEM cells. Whereas T-cell-tropic clones showed extensive variability from patient to patient, the macrophage-tropic clones from different individuals were remarkably homogeneous. These results might suggest that only certain related patterns of V3 sequences can mediate macrophage tropism. However, our mutational analyses indicated that the opposite was true

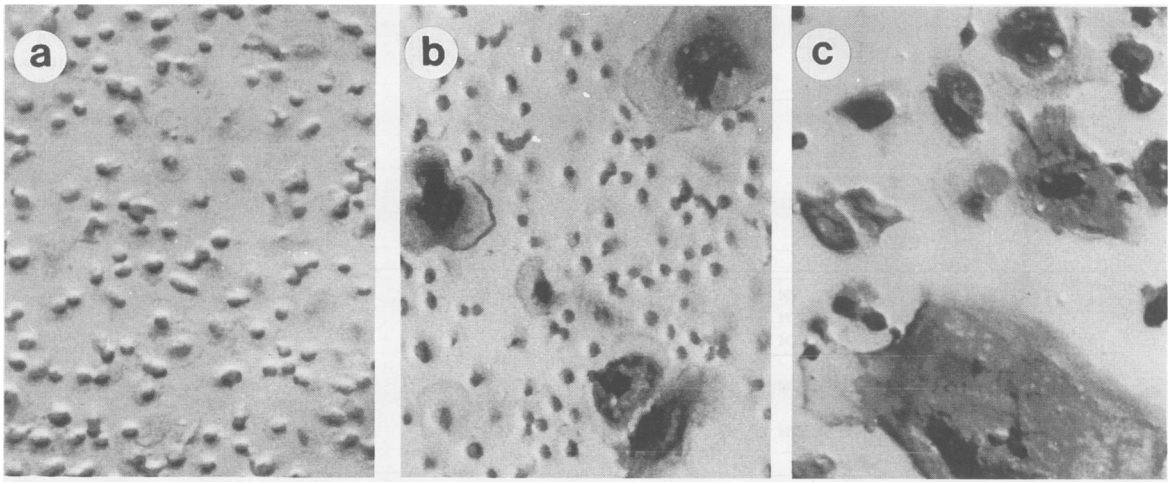

FIG. 5. Infection of macrophage cultures by HIV. Seventeen days after infection, cells were fixed and stained for HIV antigens as described in Materials and Methods. (a) Mock-infected control cells; (b) cells infected with macrophage-tropic HIV clone 147; (c) cells infected with macrophage-tropic HIV isolate BA-L. Multinucleated cells were seen only in virus-infected cultures and always had detectable HIV antigens. The number of multinucleated cells was 10 to 100% of the number of HIV antigen-positive cells, depending on the virus strains used. Infection with dilutions of virus inocula showed a directly proportional decrease in the number of multinucleated cells and the number of HIV antigen-positive cells (data not shown).

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(Fig. 3 and 4). In T-cell-tropic clones, T-cell tropism could be eliminated with concomitant production of high infectivity and replication in macrophages with as few as two or three V3 region amino acid substitutions derived from the JR-CSF sequence (clones 126, 147, and 148). The resulting V3 region sequences of these clones were not at all similar to the consensus sequence found in the macrophage-tropic patient isolates (Fig. 2). Since macrophage tropism in vitro did not appear to be restricted to only one type of V3 region sequence, there must be additional factors maintaining the sequence homogeneity of these isolates in vivo in different patients. It is possible that macrophage tropism in vivo is mediated only by a small subset of V3 sequences which can mediate macrophage tropism in vitro. Alternatively, patient-derived macrophage-tropic viruses might be more homogeneous if they were the predominant viruses transmitted between individuals during infection. Macrophage-tropic viruses do not fuse infected T lymphocytes extensively, and therefore the infected PBMC population might be more viable in vivo. Such persistently and/or latently infected cells should survive in the circulation, and their viruses could be transmitted if these cells were introduced into another person. Furthermore, if infection by these viruses did tend to become latent, the viral genomes might remain more homogeneous in each individual because of less replicative activity. These possibilities do not imply that macrophage-tropic 1022-negative viruses are found only at early times after HIV infection. In fact, macrophage-tropic viruses do not appear to be eliminated during the course of the disease (24), and many of our isolates come from patients with symptomatic AIDS (Table 1).

In contrast to the macrophage-tropic viruses, the V3 regions of T-cell-tropic viruses with high syncytium-inducing ability were highly diverse. This sequence diversity appeared to be generated independently in each individual, as variability of clones from each individual was low compared with variability between different individuals. The role of T-cell-tropic viruses in transmission of HIV is still unclear. If highly pathogenic T-cell-tropic viruses were readily transmitted between individuals, one might expect a decreasing incubation period for AIDS as the epidemic progresses. However, this has not been observed (20, 28). Preferential transmissibility of certain macrophage-tropic isolates compared with more pathogenic T-cell-tropic isolates might be one explanation for this finding. However, the data are also consistent with the possibility that the primary immune response after HIV infection of an individual might be more efficient in eliminating T-cell-tropic viruses than in eliminating macrophage-tropic viruses.

The consensus sequence of the entire group of clones from macrophage-tropic isolates from our nine patients (Fig. 2) was also quite similar to the consensus observed in a group of 245 V3 region sequences published earlier (19). However, in this previous study, no biological information on cell tropism was presented. Thus, the sequence differences between macrophage-tropic and T-cell-tropic viruses were not recognized. Nevertheless, on the basis of seroreactivity with V3 region peptides, these authors suggested that a majority of infected individuals appeared to be infected with viruses that had sequences similar to their overall consensus sequence. In conjunction with our data, this result supports the conclusion that most patients are at one time infected with the prototype macrophage-tropic HIV strains. This might be precisely at the time of initial exposure to HIV, because previous studies found that early in the disease course, HIV clones with V3 regions that have sequences nearly identical to this consensus sequence were detected (26, 31, 32). Furthermore, in some of these patients, increasing diversity of V3 sequences was observed with time. Two recent studies have suggested that viral selection and clonal restriction occur at the time of virus transmission between individuals (33, 34). It will be of interest to examine the cell tropism of the most commonly transmitted sequences in these pairs of individuals.

The V3 region is known to be an important site for a strong type-specific neutralizing antibody epitope(s) (12, 22). The present results on homogeneity and macrophage tropism of the most commonly occurring V3 region sequences suggest that these sequences from the macrophage-tropic clones would be excellent targets for an immunoprotective vaccine. Indeed, if these viruses are the main viruses involved in initial spread from person to person, a vaccine directed at these critical V3 region sequences might be effective in blocking primary infection. Since there is limited heterogeneity of these sequences, it might be feasible to develop a vaccine cocktail that includes all relevant epitopes.

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