An Infectious Molecular Clone of an Unusual Macrophage-Tropic and Highly Cytopathic Strain of Human Immunodeficiency Virus Type 1

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We isolated and molecularly cloned a human immunodeficiency virus type 1 (HIV-1) strain (89.6) which is unusual because it is both macrophage-tropic and extremely cytopathic in lymphocytes. Moreover, this is the first well-characterized infectious molecularly cloned macrophage-tropic HIV-1 strain derived from peripheral blood. HIV-1 89.6 differs markedly from other macrophage-tropic isolates within the envelope V3 region, which is important in determining cell tropism and cytopathicity. HIV-1 89.6 may thus represent a transitional isolate between noncytopathic macrophage-tropic viruses and cytopathic lymphocyte-tropic viruses.

Human immunodeficiency virus type 1 (HIV-1) strains may be divided into isolates which are exclusively lymphocyte tropic and those which are macrophage tropic, i.e., able to replicate in lymphocytes and macrophages, respectively. Isolates also vary in the ability to kill lymphocytes, and with few exceptions the macrophage-tropic isolates previously described are noncytopathic in lymphocytes (2, 4, 7, 20, 26). Studies of the molecular basis for macrophage infection have relied principally on a small number of infectious molecular clones isolated from the central nervous system (11, 15, 22). Strains isolated from the central nervous system, however, appear to constitute a distinct group which differ from peripheral blood isolates in having special patterns of target cell tropism and replication kinetics and reduced cytopathogenicity (2, 3). In addition, several partial or biologically inactive clones of blood or tissue origin have been described previously (9, 12, 27), but noninfectious clones do not allow precise correlation of genetic and biological properties present in native viruses.

To address the genetic basis for diversity among macrophage-tropic isolates, we generated an infectious molecular clone (p89.6) of a highly macrophage-tropic strain (HIV-1 89.6) from the peripheral blood of an individual with AIDS but without neurological disease. We selected this isolate because it has a phenotype which has not previously been studied but may be important in viral pathogenesis in that it is markedly cytopathic in primary lymphocytes and replicates efficiently in macrophages. Moreover, this is the first well-characterized biologically active, molecularly cloned highly macrophage-tropic HIV-1 strain derived from peripheral blood.

Origin of strain 89.6. HIV-1 89.6 was obtained from peripheral blood mononuclear cells (PBMC) from a 47-year-old man who had emigrated to Philadelphia from Jamaica 15 years earlier. At the time of virus isolation he had received no antiviral therapy and had advanced immunodeficiency, with opportunistic infections and ≤ 10 CD4-positive cells per

mm². He had no neurological disease at any time during his illness.

The patient's PBMC, containing both lymphocytes and monocytes, were cocultured with phytohemagglutinin (PHA)-interleukin 2 (IL-2)-stimulated seronegative PBMC. Viral p24 antigen was detected after 1 week, and supernatant was tested for replication in primary macrophages and lymphocytes. Monocyte-derived macrophages (MDM) were isolated from PBMC by a two-step adherence method and maintained as previously described (4). These cultures were essentially devoid of lymphocytes by immunofluorescence and other criteria (4), and they were infected ~1 week after isolation. Peripheral blood lymphocytes (PBL) were isolated from PBMC by depletion of adherent cells as described previously (4), stimulated with PHA for 3 days, and maintained with IL-2. The initial isolate was found to replicate efficiently in both PBL and MDM (data not shown). Because it was likely to contain a mixture of related viruses, we first tested whether brief culture in mixed PBMC would select among coexisting strains and affect the ability of 89.6 to replicate in MDM. The initial isolate was passaged onto fresh PBMC, and after 3 weeks of culture the cell-free supernatant was found to productively infect MDM, indicating that this brief passage *in vitro* had not selected against macrophage-tropic variants. The cell-free supernatant therefore was harvested as 89.6 primary isolate (89.6[pi]) working stock, and the genomic DNA was extracted for cloning. Because monocytes were not specifically depleted from either patient or target PBMC, the cell of origin of the virus (monocyte versus lymphocyte) is not known. However, by visual inspection the majority of cells in the PHA-IL-2-stimulated cultures were lymphocytes, and, since the DNA used for cloning was extracted from nonadherent cells rather than an adherent subfraction, the cloned virus most likely derived from lymphocytes in the culture.

Generation of an infectious molecular clone. High-molecular-weight DNA was isolated by standard methods and subjected to restriction endonuclease digestion followed by Southern blot (18). This showed that *Sac*I released a single HIV-1 fragment of ~9.1 kb, which is compatible with

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conserved *SacI* sites repeated in each long terminal repeat (LTR) (+493 and +9576, based on the numbering system of HXB2 as in reference 13). We therefore generated a subgenomic library of size-fractionated DNA. DNA was digested to completion with *SacI* and separated on a 10 to 40% sucrose gradient into 72 fractions. An aliquot from each was analyzed by Southern blot, and the two fractions with the strongest HIV-1 signal were ligated into *SacI*-cut phage arms and packaged in vitro (Gigapak Gold; Stratagene Cloning Systems). Approximately 150,000 plaques were screened by colony hybridization, yielding three full-length HIV-1-containing recombinant phages. By restriction mapping they were closely related, sharing several unique restriction sites, but also had some differences which indicated that they were distinct (data not shown). One of these was selected for further characterization, and the *SacI* insert was subcloned into a plasmid to yield p43.

Because p43 was cloned with *SacI* sites located in R, the termini of each LTR were truncated. We reconstructed the LTRs in vitro by utilizing U3 sequences from the 3' region of p43 to complete the 5' LTR and U5 sequences from the 5' portion of p43 to complete the 3' LTR. An ~560-bp *KpnI-SacI* fragment from the 3' portion of p43 and an ~930-bp *SacI-PstI* fragment from the 5' portion of p43 were ligated into a plasmid (Bluescript; Stratagene) to form a single complete LTR construct. This was then digested with *SacI* and the 9.1-kb *SacI-SacI* fragment from p43 was inserted, forming a complete provirus (p89.6) with short flanking sequences derived from internal coding regions. Transfection of p89.6 into rhabdomyosarcoma cells (24) resulted in the release of infectious virus, indicating that the in vitro reconstruction had yielded a biologically active infectious proviral clone. Virus was then amplified in CEMX174 cells (17) to generate stocks of molecularly cloned HIV-1 89.6 (89.6[mc]).

Biological characterization of strain 89.6. To examine relative tropism for primary cell types, we compared 89.6[mc] and 89.6[pi] with cloned virus HXB2 (21) derived from the prototype non-macrophage-tropic strain IIIB (Fig. 1). Inocula were standardized on the basis of infectious units, and infections were carried out as previously described (4). In MDM, both 89.6[pi] and 89.6[mc] replicated efficiently and produced sustained levels of p24 antigen, while HXB2 replicated poorly or not at all (Fig. 1A). Consistent with results with other macrophage-tropic isolates (4), MDM from different donors varied in the absolute levels to which viral replication was supported. Both 89.6[pi] and 89.6[mc] replicated rapidly and to high titer in PBL, reaching peak levels which were consistently greater than those of HXB2 (Fig. 1B). Thus, 89.6[pi] and 89.6[mc] are macrophage tropic, replicating in both primary cell types, and have "rapid/high" (6) replication kinetics in lymphocytes.

Neither 89.6[pi] nor 89.6[mc] productively infected the monocytoid U937 cell line (data not shown), which is consistent with the finding that U937 cells do not resemble primary monocytes and macrophages with respect to HIV-1 cell tropism (4). Similarly, neither SUP-T1 nor CEM lymphocytoid cells were permissive for 89.6, while HXB2 replicated in both. Thus, 89.6 is similar to many primary isolates and most macrophage-tropic strains in that it is restricted in transformed cell lines, including monocytoid cells (4, 20). The CD4-positive hybrid cell line CEMX174 (17), however, was highly permissive for both 89.6[pi] and 89.6[mc] (Fig. 1C).

Many lymphocyte-tropic isolates produce syncytia in PBL, but most macrophage-tropic strains, including other

FIG. 1. Replication of HIV-1 strains 89.6[pi] (○), 89.6[mc] (□), and HXB2 (△) in primary MDM (A), primary PBL (B), and the transformed CEMX174 cell line (C). MDM which had been maintained in culture for 1 week, PHA-stimulated PBL, and CEMX174 cells were inoculated with cell-free virus (approximate multiplicity of infection, 0.05 infectious units per cell), washed three times, and maintained as previously described (4). Culture supernatant was tested for viral p24 antigen production.

molecularly cloned macrophage-tropic isolates, are noncytopathic in these cells (4, 20). In marked contrast, both cloned and uncloned 89.6 produced a striking cytopathic effect in PBL (data not shown). The extent of syncytium formation was consistently greater than that induced by HXB2, and rapid viral replication was followed by rapid death of the culture. 89.6 also induced formation of extremely large and numerous syncytia in CEMX174 cells (data not shown). A notable difference between 89.6 and HXB2 is that HXB2 killed CEMX174 cells through a combination of syncytium formation and single cell killing (6), while 89.6 induced dramatic cell fusion and rapid cell death but showed little evidence of individual cell killing (data not shown). HIV-1 89.6 also induced formation of particularly striking syncytia in MDM, and immunofluorescent staining (4) showed extensive viral antigen accumulation in the multinucleate cells (Fig. 2). In addition, while most macrophage-tropic HIV-1 strains do not kill infected MDM (4), giant cells in 89.6-infected cultures occasionally showed ballooning, degeneration, and death (data not shown).

FIG. 2. Morphology of HIV-1 89.6-infected MDM. MDM were infected with 89.6[mc] and 27 days later were fixed and stained by a modification of the Wright method (A and B) or stained for viral antigens by immunofluorescence (C and D). A and C, uninfected MDM; B and D, MDM infected with 89.6[mc]. Magnifications, $\times 250$ (A and B) and $\times 200$ (C and D).

Sequence of p89.6. Because host cell tropism, cytopathicity, and other biological properties have been mapped to genes in the 3' half of the genome, we determined the DNA sequence of p89.6 from the beginning of the *vif* gene through the 3' LTR (4.7 kb). HIV-1 89.6 possesses intact open reading frames for the *vif*, *vpr*, *vpu*, *tat*, *rev*, *nef*, and *env* genes. There is 92.5% overall nucleotide homology within this region compared with the HXB2 clone of cytopathic non-macrophage-tropic strain IIIB (14), and it is not significantly different from either other lymphocyte-tropic strains or macrophage-tropic isolates such as JRFL, SF162, Bal, and ADA. Nucleotide and predicted amino acid homologies of specific genes with those of the prototypes HXB2 and JRFL are shown in Table 1. Within *env*, regions of significant divergence are concentrated in recognized hypervariable regions (23). All 19 cysteines of gp120 are conserved, as are the residues critical for CD4 binding (16) and the hydro-

phobic fusion region of gp41 (8). Predicted amino acid homology of gp120 from macrophage-tropic isolates JRFL, SF162, Bal, and ADA and lymphocyte-tropic strains MN, SF2, and HXB2 ranges from 88 to 91%, but there is no greater homology with either group.

Since several studies have indicated that regions of the envelope which include the third hypervariable region (V3) of gp120 play a critical role in host cell tropism (1, 7, 9, 15, 22, 27, 29), we compared this region of p89.6 with those of several macrophage-tropic and non-macrophage-tropic clones as well as the consensus sequence described by LaRosa et al. (10) (Fig. 3). Within this 35-residue region, p89.6 diverges from the consensus at eight sites, which stands in contrast to the sequences of the other macrophage-tropic clones, which are very similar to the consensus sequence. Pairwise distance comparisons (5) of the V3 sequences among these strains showed that 89.6 was no

TABLE 1. Nucleotide and predicted amino acid homologies between HIV-1 89.6 and two prototype cloned HIV-1 strains

Region	Homology ^a between 89.6 and:			
	HXB2 ^b		JRFL ^c	
	Nucleotide	Amino acid	Nucleotide	Amino acid
<i>env</i>	91.6	90.4	92.2	91.5
gp120	90.6	89.2	91.6	90.6
gp41	93.2	92.2	93.0	92.8
<i>tat</i>	94.2	94.2	95.1	94.1
<i>rev</i>	92.9	94.0	93.5	94.0
<i>vif</i>	96.0	96.4	95.3	93.2
<i>vpr</i>	94.5	94.9 ^d	93.8	93.8
<i>vpu</i>	86.4	82.5 ^d	91.4	92.5
<i>nef</i>	90.2	90.9 ^d	89.4	93.1
LTR	92.7		90.9	

^a Homology is expressed as a percentage (13, 14).

^b Clone of the non-macrophage-tropic syncytium-inducing isolate IIIB.

^c Cloned macrophage-tropic non-syncytium-inducing isolate.

^d Lacks intact open reading frame, while 89.6 and JRFL possess intact open reading frames for all genes listed.

more closely related to the macrophage-tropic isolates than to cytopathic non-macrophage-tropic strains and indicated that 89.6 is more distant from the consensus sequence than any other macrophage-tropic strain (data not shown). Because 89.6 may have originated in Jamaica, we compared the V3 region with that of Caribbean isolate RF (13) but found it to be no more closely related.

In addition, 89.6 is distinct from other macrophage-tropic isolates in several V3 sequence motifs which have been proposed to play a role in determining tropism. Westervelt et al. (27) noted a Tyr at position 283 and an acidic or Ala residue at position 287 in macrophage-tropic strains. 89.6 possesses the Tyr-283 but has an Asn at position 287. Fouchier et al. (7) found basic residues at position 273 and/or 287 in cytopathic isolates which failed to replicate in macrophages but acidic or uncharged residues in macrophage-

tropic strains. They also found this region to be more positively charged overall in non-macrophage-tropic cytopathic isolates. 89.6 has uncharged residues at both of these positions but carries an extremely strong net positive charge (arginines compose 8 of 35 residues).

Biological significance of HIV-1 89.6. To our knowledge, 89.6 is the first macrophage-tropic strain described which is highly cytopathic in T cells and is the first well-characterized biologically active molecularly cloned macrophage-tropic HIV-1 strain derived from peripheral blood. While highly cytopathic in lymphocytes, 89.6 retains the host range of macrophage-tropic isolates in that it fails to replicate in most transformed cell lines. The clone was generated after minimal passage in vitro and accurately reflects the primary isolate in cell tropism, replication kinetics, and cytopathogenicity.

Several studies have reported that individuals with asymptomatic infection harbor mainly strains which replicate in both macrophages and lymphocytes but which do not induce syncytium formation in PBL and that progression to clinical disease is characterized by the emergence of variants that are highly cytopathic in PBL but no longer productively infect macrophages (19, 20). Whether cytopathic variants are derived from noncytopathic isolates present during the asymptomatic period, or represent the emergence of strains previously suppressed, is unknown. Moreover, it is not known whether the emergence of cytopathic variants is the cause of T-cell destruction or merely reflects immunodeficiency and loss of viral control. The relationship between macrophage-tropic noncytopathic variants and lymphocyte-tropic cytopathic variants is not presently understood, however, and represents an important gap in our understanding of viral pathogenesis. We speculate that 89.6 may be a transitional isolate which retains the ability for efficient replication in macrophages while having acquired syncytium-inducing cytopathic characteristics.

Several studies have shown that tropism for macrophages is mainly determined by portions of the envelope, including the V3 region (9, 15, 22, 27). The V3 sequences of macrophage-tropic strains previously characterized are closely related to the consensus (10), while those of cytopathic strains and those which fail to productively infect macrophages are more diverse. It is not known whether this truly reflects limited diversity of macrophage-tropic viruses or is the result of the small sample of macrophage-tropic clones that have been studied in detail. It is therefore of interest that the V3 region of 89.6 is quite distant from the consensus sequence and that in this region 89.6 is the most divergent macrophage-tropic isolate yet characterized. In addition, several motifs within the V3 region have been proposed to play a role in the determination of macrophage tropism (7, 27), but 89.6 does not demonstrate these patterns. These data indicate that there are multiple V3 patterns compatible with productive macrophage infection and, consistent with recent reports (15, 22, 25, 28), suggest that additional regions may be involved in determining tropism.

These results show that the range of biological and genetic characteristics of macrophage-tropic viruses is broader than previously recognized and indicate that studies of macrophage infection require the use of macrophage-tropic strains of differing phenotypes, such as 89.6, and isolated from a variety of sources. As an infectious molecularly cloned primary isolate with the unique combination of macrophage tropism and T-cell cytopathicity, HIV-1 89.6 will shed light on the relationship between noncytopathic macrophage-

virus	V3 loop sequence	M-tropic	syncytia
ZR6	---YK---Q-TP---L-Q-L---RGRTKI-G---	no	yes
HXB2	---R-R-QR---V-I-K---NM---	no	yes
HN	---Y-K-R---KN---T---	no	yes
SF2	---S---Y---H---R---K---	no	no
JRCSF	---S---	no	no
consensus	263 CTRPNNMTRKSIHI · GPGRFYTTGEIIGDIRQAHC 297		
JRFL	----- · -----	yes	no
ADA	----- · -----	yes	no
Bal	-----L-----	yes	no
YU-2	-----N-----L-----	yes	no
SF162	-----T-----A-D-----	yes	no
89.6	-----RRLS · -----ARRN-----	yes	yes

FIG. 3. Comparison of predicted amino acid sequences of the V3 loop (the principal neutralizing domain). The V3 consensus sequence as determined by LaRosa et al. (10) is shown at the center, with aligned sequences of non-macrophage-tropic strains grouped above it and those of macrophage-tropic isolates grouped below it (13). Sequences within each group are ordered with respect to the consensus, according to their degree of divergence (5). Phenotype is indicated to the right; syncytium-forming ability refers to infection of PBL or permissive T-cell lines. Some of the clones are not biologically active, and phenotype is based on the uncloned isolate or on recombinant viruses containing the envelope region of the indicated clone. Dots indicate gaps; dashes indicate conserved amino acids.

tropic and cytopathic lymphocyte-tropic groups of strains which are relevant to pathogenesis in vivo.

Nucleotide sequence accession number. The sequence described in this article has been submitted to the GenBank data base under accession number M96155.

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