Infection of Primary Human Microglia and Monocyte-Derived Macrophages with Human Immunodeficiency Virus Type 1 Isolates: Evidence of Differential Tropism

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To ascertain whether viruses present at the time of primary viremia can infect the central nervous system and to determine if microglial tropism is distinct from tropism for monocyte-derived macrophages (MDM), 27 human immunodeficiency virus type 1 (HIV-1) isolates obtained from acutely infected individuals, as well as laboratory strains, were assayed for their ability to replicate in primary adult microglial cultures and in MDM. Most of the isolates replicated equally well in both microglia and MDM, but several isolates replicated preferentially in one of the two cell types, differing by as much as 40-fold in p24^{agg} production. This indicated that while MDM and microglial tropism overlap, a subset of isolates is particularly tropic for one of the two cell types. One isolate was further adapted to microglia by 15 sequential passages, raising the peak p24 concentration produced by 1,000-fold. In addition, the passaged virus induced marked cytopathologic changes (vacuolization and syncytium formation) in infected microglial cultures. Sequence comparison of the V3 loop of unpassaged and multiply passaged virus revealed amino acid changes shown to be associated with isolates from patients with HIV dementia. Our data support the hypothesis that HIV-1 infection can be established in the central nervous system by viruses present early in HIV infection, that some of these viruses are particularly tropic for microglia, and that adaptation in this cell type can result in the selection of a pool of predominantly microglia-tropic (neurotropic) viruses.

AIDS dementia complex or human immunodeficiency virus (HIV) dementia (HIVD), the primary central nervous system (CNS) disorder associated with the HIV-1, affects 20 to 30% of infected individuals, although neuropathological changes may be present in an even greater proportion (5, 19, 36, 42). This syndrome is characterized by a range of neurological symptoms involving both motor function and cognitive abilities, including slowed movements, tremors, behavioral changes, and memory loss. Immunohistochemical, in situ hybridization, and in situ PCR studies of brain sections from infected individuals have demonstrated that microglia and microglia-derived giant cells are the predominant cells expressing HIV-1 genes (3, 27, 53) and, at the very least, are responsible for the persistence of virus in the CNS.

The precise pathogenesis of HIVD is not well understood (14). Microglia may mediate CNS disease by (i) secreting neurotoxic substances such as tumor necrosis factor alpha, platelet-activating factor, and arachidonic acid metabolites, (16–18, 55); (ii) shedding viral proteins such as gp120 or tat, both of which reportedly have neurotoxic properties (29, 35, 37, 46); or (iii) mediating the direct infection of astrocytes or oligodendrocytes, which are capable of harboring a restricted infection with HIV-1 (1, 28, 43, 49).

Recent studies have brought to light the crucial role of primary viremia in the pathogenesis of HIV-1 infection (2, 11, 21, 22). During this initial burst of replication, the virus disseminates throughout the body and establishes a persistent

infection at many sites, including the CNS (4). Early brain invasion may be a critical step for the establishment of a chronic infection in the CNS, ultimately leading to the more progressive HIVD. In support of this model, analysis of HIV sequences amplified directly from brain tissue has shown that the virus quasi-species residing within the CNS are considerably more homogeneous than those present in lymphoid tissue (15, 41), suggesting that virus entering the brain early in infection may be sequestered and evolve independently of the virus population present in the peripheral circulation and lymphoid organs.

The viral elements responsible for neurotropism are likely to be complex. Many HIV-1 isolates recovered from the brain or cerebrospinal fluid have a monocyte-derived macrophage (MDM)-tropic phenotype (7, 34, 40), and experimental infection of cultured microglia (the resident CNS macrophages) indicates that microglia are susceptible to infection with macrophage-adapted but not T-cell-tropic isolates (50). However, although tropism for MDM appears to be a correlate for growth in microglia, there have not been any systematic studies exploring relative viral replication in the two cell types.

Previous studies of microglial tropism have for the most part used laboratory-adapted or cloned viruses that may have undergone in vitro selection prior to analysis (24, 33, 47, 50). Since HIV involvement of the CNS can occur early (4), we chose to examine the ability of HIV-1 isolates obtained during primary viremia or early stages of infection to replicate in both MDM and microglial cultures. These viruses are more homogeneous than those isolated late in the course of infection (58). In addition, we sequentially passaged several primary isolates in microglia to evaluate the ability of these isolates to adapt to this cell type. Our results indicate that most acute HIV-1 iso-

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TABLE 1. HIV-1 primary isolates used in this study

Isolate designation	Description ^a	Phenotype ^b	Passage history ^c	Source ^d
SUMA	Acute	NSI	2	1
HOBR	Acute	NSI	2	1
BORI	Acute	NSI	2	1
FASH	Acute	NSI	2	1
WEAU	Acute	SI	2	1
AD-6	Acute	NSI	2	2
301657	5 mo p.i.	ND	6	3
301727	3 mo p.i.	ND	6	3
302056	Pp 1 wk	NSI	6-7	3
302151	Pp 1 wk	SI	6-7	3
302077	Pp >8 wk	SI	6-7	3
302144	Pp >8 wk	NSI	6-7	3
AL-6244	Acute	NSI	2	4
AM-3107	Acute	NSI	3	4
26-6884	Acute	NSI	2	4
AJ-4331	Acute	NSI	3	4
AU-3040	Acute	NSI	3	4
AD-8874	Acute	NSI	2	4
AM-5304	Acute	NSI	3	4
AP-6658	Acute	NSI	2	4
AQ-3962	Acute	NSI	3	4
AQ-4394	Acute	NSI	3	4
AQ-4697	Acute	NSI	2	4
AJ-3879	Acute	NSI	2	4
AQ-5945	Acute	NSI	4	4
AQ-6577	Acute	NSI	3	4
AQ-6809	Acute	NSI	3	4

^a Viruses isolated from adult patients during primary (acute) HIV-1 infection, at the indicated time after seroconversion, or from infants (1 to 8 weeks post-partum [Pp]).

^b NSI, non-syncytium inducing; SI, syncytium inducing; ND, not determined.

^c Viruses were isolated from plasma or peripheral blood lymphocytes from infected patients and amplified two to seven times by addition of PHA-stimulated PBMCs.

^d Virus isolates were obtained from the following sources: 1, G. Shaw, University of Alabama, Birmingham, Ala.; 2, D. Ho, Aaron Diamond AIDS Research Center, New York, N.Y.; 3, AIDS Research and Reference Reagent Program, Division of AIDS, and Gregory Melchers, HIV clinical isolate panel, National Institute of Allergy and Infectious Diseases; 4, L. Perrin, Geneva University Hospital, Geneva, Switzerland.

lates can replicate in microglia and that a subset of such isolates may indeed be preferentially microglia tropic.

MATERIALS AND METHODS

Virus isolates. The source, passage history, and phenotype of the primary HIV-1 isolates used in this study are listed in Table 1. Viruses were isolated from sera or peripheral blood mononuclear cells (PBMCs) of individuals with primary HIV-1 infection by cocultivation with phytohemagglutinin (PHA)-stimulated PBMCs from normal donors. The isolates were further expanded in our laboratory once or twice in PHA- and interleukin-2-stimulated PBMCs obtained from seronegative volunteers to prepare working stocks. The passage number indicates the total number of additions of fresh PHA blasts to the original patient sample. Virus strains, HIV-1_{JRCSE}, HIV-1_{JFL}, HIV-1_{SF162}, HIV-1_{IIIB}, HIV-1_{ADA}, HIV-1_{NL4-3}, HIV-1_{YU-2}, and HIV-1_{Bal}, were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases. HIV-1_{89.6}, HIV-1_{NDK}, and HIV-1_{DV} were obtained from Ron Collman (University of Pennsylvania, Philadelphia, Pa.), Ivan Hirsch (Institut National de la Santé et de la Recherche Médicale, Marseilles, France), and Michael McGrath (University of California, San Francisco, Calif.), respectively. Laboratory isolate stocks were prepared in normal PHA-stimulated PBMCs, except for HIV-1_{IIIB} and HIV-1_{89.6} which were propagated in CEMx174 cells.

Cell isolation and culture. (i) **MDM cultures.** Whole blood or buffy coats collected from normal healthy donors or buffy coat preparations (American Red Cross, Philadelphia, Pa.) were centrifuged over a Ficoll gradient (1,200 × g for

FIG. 1. Growth curve of HIV-1 laboratory isolates. Microglial cultures were infected with strains HIV-1_{JRCSE} (○), HIV-1_{JFL} (■), HIV-1_{NL4-3} (△), HIV-1_{YU-2} (◆), or HIV-1_{NDK} (□), and viral replication was monitored by measuring p24^{agg} production for 28 days. Viruses with the MDM-tropic (NSI) phenotype (HIV-1_{JFL}, HIV-1_{YU-2}) replicated 100 to 1,000 times better in the microglial cultures than did the T-cell-tropic viruses HIV-1_{NDK} and HIV-1_{NL4-3}. The T-cell-tropic isolate HIV-1_{JRCSE} exhibited variable tropism in different experiments (see Table 2).

20 min), and the mononuclear cell fraction was collected and washed in RPMI 1640. The cells were resuspended in RPMI 1640 containing 10% human serum and seeded onto plastic tissue culture flasks for 1 h to allow monocytes to adhere; the nonadherent cells were then removed by extensive washing. Monocytes were removed from the flask with 530 μM EDTA-phosphate-buffered saline (PBS) and seeded onto 24-well tissue culture dishes. Cells were maintained in M/M medium, composed of Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum, 5% giant cell tumor supernatant (IGEN Inc., Rockville, Md.), 1 mM sodium pyruvate, and 50 μg of gentamicin per ml. The cultures were incubated at 37°C under 5% CO₂ for 5 to 7 days prior to infection.

(ii) **Microglial cultures.** Fresh human brain tissue was obtained from donors undergoing temporal lobectomy surgery for medication-resistant epilepsy. Cells were isolated by a previously described method adapted from that of Yong and Antel (1, 50, 57). Briefly, tissue was dissected free of meninges and large blood vessels and finely minced. The minced tissue was then digested for 30 min at 37°C in DMEM containing 0.25% trypsin, 50 μg of DNase per ml, and 50 μg of gentamicin per ml. The resulting single-cell suspension was filtered through a 100-μm nylon mesh, and the cells were pelleted by centrifugation. The pellet was then resuspended in 30% Percoll-PBS and centrifuged at 17,500 × g in a Sorvall SS34 fixed-angle rotor for 30 min to form a gradient. The upper layer of the

TABLE 2. Growth of laboratory HIV-1 isolates in human microglial cultures

HIV-1 strain	Tropism ^a	Growth in microglia ^b
NL4-3 ^c	T	—
NDK ^c	T	—
JRCSE	T	-/+ ++
IIIB	T	-/+ ++
89.6 ^c	M	+++
Bal	M	+++
DV	M	++
JR-FL	M	++
YU-2 ^c	M	++
ADA	M	++
SF-162	M	++

^a T, replicates in PBMCs; M, replicates in MDM and PBMCs.

^b Microglial cultures were infected with virus inocula (10 to 20 ng of p24^{agg}) as indicated in Materials and Methods. Viral replication was determined by peak p24^{agg} antigen production between days 14 and 28 of infection and is summarized as follows: —, <0.1 ng/ml; +, 0.1 to 1 ng/ml; ++, 1 to 10 ng/ml; +++, >10 ng/ml; -/+, ++, p24^{agg} values varied from <0.1 to >10 ng/ml among individual experiments.

^c Molecularly cloned strain.

FIG. 2. Growth of primary HIV-1 isolates in MDM and microglia. MDM and microglial cultures were infected with virus inocula containing equivalent p24^{agg} titers (50 ng/2 × 10⁵ cells) as described in Materials and Methods. Culture supernatants were replaced weekly and assayed for the presence of p24^{agg} antigen by ELISA. Several different patterns of growth in the two cell types were observed.

gradient, containing myelin debris, was discarded, while the central portion, containing predominantly microglia and oligodendrocytes, was collected and washed twice in PBS. The mixed-cell culture was then resuspended in DMEM supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, penicillin, and streptomycin and incubated at 37°C under 10% CO₂. The nonadherent oligodendrocytes were removed from the adherent microglia 24 to 48 h after isolation by washing. Microglia were propagated in M/M medium for 7 to 10 days before being reseeded in 24-well tissue culture wells for infection experiments.

Virus infections. MDM and microglia cultures were seeded onto 24-well tissue culture plates at a density of 2 × 10⁵ to 2.5 × 10⁵ per well and infected with cell-free virus supernatant that was standardized for each experiment by p24^{agg} antigen concentration. The cultures were infected for 12 to 18 h at 37°C, the inoculum was removed, and the cells were washed twice with DMEM and then maintained in 1 ml of M/M medium. Culture supernatants were replaced weekly and assayed for the presence of virus by p24^{agg} enzyme-linked immunosorbent assay (ELISA; DuPont/NEN).

Indirect immunofluorescence. Microglia or MDM were seeded onto four- or eight-well Permanox chamber slides (Nunc) and infected as described above. Microglia were positively identified by the addition of 1 µg of Di-I-Acylated LDL (International Biotechnologies Inc.) per ml to the culture medium followed by incubation for 2 to 3 h at 37°C to allow internalization. The cells were also incubated with 0.2 mg of nucleus-specific Hoechst 33342 stain (bisbenzamide; Molecular Probes, Inc., Eugene, Ore.) per ml. The cultures were washed in PBS, fixed in 4% paraformaldehyde, and mounted with Vectashield (Vector Labs, Burlingame, Calif.). The cells were photographed with a Leitz Aristoplan microscope with an epifluorescent source.

Virus passaging. Selected virus isolates were sequentially passaged in microglia cultures by transferring supernatant from 21- to 28-day-old infected cultures to freshly prepared uninfected microglial cultures. Virus replication was monitored by p24^{agg} antigen production.

PCR and sequencing. HIV-1-infected PBMCs or microglia were lysed for 2 h at 58°C in buffer consisting of 100 mM KCl, 0.1% Nonidet P-40, 20 mM Tris (pH 8.4), and 500 µg of proteinase K per ml and then subjected to heat inactivation at 95°C for 15 min. The V3 loop region was PCR amplified from the lysates by using the nested primer sets described by Wolinsky et al. (56). The resulting PCR products were purified and sequenced with an ABI 373A automated fluorescence sequencer (Molecular Biology Core Facility, Children's Hospital of Philadelphia, Philadelphia, Pa.).

RESULTS

Infections with laboratory-passaged isolates. To date, only a limited number of isolates have been studied for their growth potential in microglia (24, 50). To confirm and extend the previous findings, we examined a panel of 11 cloned and uncloned T-cell-tropic and MDM-tropic HIV-1 laboratory-passaged isolates for their ability to replicate in this cell type. Representative growth curves of five of these isolates are depicted in Fig. 1, and the results for all isolates are summarized in Table 2.

All of the MDM-tropic laboratory-passaged isolates produced high levels of p24^{agg} antigen in the microglial cultures. Conversely, the T-cell-tropic viruses HIV-1_{NL4-3} and HIV-1_{NDK} replicated poorly, consistent with prior reports (24, 50). However, in some experiments, several of the T-cell-tropic viruses, HIV-1_{IIIB} and HIV-1_{JRCSEF}, produced p24^{agg} antigen levels comparable to those of the MDM-tropic viruses (Fig. 1; Table 2). This variability between experiments may reflect either donor variability or the state of activation or differentiation of some of the microglial cultures. Together, these results suggested that while microglial and MDM tropism overlap, they are not necessarily indistinguishable.

Infections with primary isolates. Recent studies have suggested that the viremia that occurs during primary HIV-1 infection may be important in the dissemination and seeding of virus throughout the body, including the CNS (22). Thus, tropism for different organs, and specifically the CNS, may be defined at this stage. Furthermore, isolates obtained during primary HIV-1 infection are relatively homogeneous (58).

Twenty-seven HIV-1 isolates cultured during primary infec-

TABLE 3. Replication of HIV-1 primary isolates in microglia and MDM

Virus isolate	Mean peak p24 ^{gag} titer in ^a :		Fold difference ^b
	Microglia	MDM	
302077	3.38 ± 0.47	4.20 ± 0.81	0.15
AJ-4331	2.43 ± 1.5	2.47 ± 1.1	0.87
301727	4.30 ± 0.23	4.32 ± 0.40	0.95
WEAU	2.83 ± 0.29	2.84 ± 1.5	0.95
302144	3.34 ± 1.0	3.31 ± 1.1	1.07
AD-6	3.18 ± 1.8	3.12 ± 1.1	1.15
FASH	4.49 ± .43	4.41 ± 0.52	1.20
BORI	4.02 ± 1.8	3.89 ± 0.28	1.35
AQ-4394	3.66 ± 0.69	3.52 ± 0.34	1.38
AQ-6809	3.29 ± 0.87	3.04 ± 0.84	1.78
AM-5304	2.88 ± 1.2	2.61 ± 0.36	1.90
AP-6658	3.77 ± 1.2	3.44 ± 0.28	2.10
AQ-3962	3.90 ± 0.87	3.53 ± 0.54	2.30
SUMA	3.41 ± 0.14	3.00 ± 1.8	2.57
302056	4.23 ± 0.47	3.80 ± 0.82	2.69
301657	4.52 ± 0.10	3.95 ± 0.89	3.70
AD-8874	4.01 ± 1.1	3.44 ± 0.58	3.70
26-6884	2.81 ± 1.5	2.21 ± 0.9	3.98
AM-3017	4.33 ± 0.59	3.61 ± 0.70	5.25
AU-3040	3.38 ± 1.0	2.47 ± 1.2	8.13
AL-6244	4.26 ± 0.75	3.26 ± 0.41	10.0
AQ-4697	3.09 ± 0.73	2.02 ± 0.90	11.7
AJ-3879	4.15 ± 0.53	3.04 ± 0.60	12.9
AQ-6577	2.85 ± 0.9	1.63 ± 0.52	16.6
HOBRR	2.99 ± 0.38	1.43 ± 0.58	36.3
AQ-5945	3.74 ± 1.1	2.13 ± 0.54	39.8
302151	1.77 ± 0.84	1.28 ± 0.37	— ^c

^a Microglia and MDM cultures were infected for 16 to 24 h with cell-free virus stocks containing equivalent p24^{gag} values (50 ng). Culture supernatants were collected weekly for 3 to 4 weeks and assayed for virus production by p24^{gag} ELISA. The geometric mean of the peak p24^{gag} titers of three individual experiments are reported as log₁₀ picograms per milliliter ± standard deviation.

^b Ratio of the geometric means of microglia p24 titer/MDM p24 titer. The fold difference represents the arithmetic value of the ratio between the two means.

^c HIV₃₀₂₁₅₁ replicated to minimal levels in each cell type, and the ratio was not calculated.

tion were assayed for their ability to replicate in both primary MDM and brain microglia. Equivalent numbers of each cell type seeded onto 24-well tissue culture dishes were infected with inocula equilibrated by measuring their p24^{gag} titers, and samples were collected at intervals throughout the entire lifetime of the cultures (≈ 30 days). Figure 2 shows the growth curves of four representative viruses in both MDM and microglia. Most isolates exhibited comparable replication in both cell types, as exemplified by isolate 302144. However, some viruses replicated preferentially in one of the two cell types. For example, isolate 302077 replicated better in MDM, while isolates AQ-6577 and AQ-5945 replicated better in microglia.

For all isolates, peak p24^{gag} production occurred between days 14 and 28 after infection. These values are shown as the geometric mean for three experiments with each cell type in Table 3, along with a ratio (microglia/MDM) of the peak concentration for each cell type. Overall, the p24^{gag} production was approximately threefold higher in the microglial cultures than in the MDM cultures, perhaps reflecting different states of activation of these two cell types (see below). The viruses could then be divided into four categories on the basis of this ratio. The majority of the isolates (16 of 27), demonstrated similar growth in both microglia and MDM, with ratios ranging

FIG. 3. Influence of passage of HIV-1 isolates in microglia on peak p24^{gag} titers. HIV-1_{BORI} and HIV-1_{89.6} were sequentially passaged in microglial cultures by transferring infected supernatants at intervals of 14 to 28 days. Virus replication in each culture was assayed by p24^{gag} ELISA of supernatants at weekly intervals, and the datum points represent the peak p24^{gag} level for each virus passage.

between 1 and <10. However, one group of four viruses had a clear preference for MDM, with a peak microglia/MDM ratio of <1, and six other viruses replicated preferentially in microglia (ratio of ≥10). Although the absolute peak p24^{gag} titer varied among experiments with each virus, as a result of donor variability, the pattern of differential tropism was maintained consistently.

These results raised the possibility that some primary viruses have a greater potential for replication within the CNS and therefore are more likely to cause CNS disease. Interestingly, two of six isolates that showed preferential replication in microglia were obtained from individuals who had developed neurological symptoms at the time of primary HIV-1 infection. Patient AJ-3879 had acute encephalitis with mental status changes that eventually resolved, and patient AL-6244 developed a cranial neuropathy prior to seroconversion. None of the individuals from whom the other 19 isolates were obtained had

FIG. 4. Comparative growth of HIV-1_{B_{ORI}} (■) and HIV-1_{B_{ORI}/14} (○). Microglia, MDM, and PHA-stimulated PBMC cultures were infected with inocula containing 4.5 ng of HIV-1_{B_{ORI}} or HIV-1_{B_{ORI}/14} as described in Materials and Methods. Culture supernatants were collected weekly for 4 weeks and assayed for p24^{gag} production. In comparison with the original virus isolate, the passaged virus replicated better in both microglia and MDM. In contrast, the original virus replicated to high titers in PBMCs whereas the microglial passaged virus replicated poorly. This experiment is representative of three or four experiments with microglia and MDM and two experiments with PBMCs.

any documented neurological problems at the time of primary infection (clinical data were not available for six individuals).

Cytopathicity in microglial cultures. A hallmark of HIV-1 infection in the brain is the presence of multinucleated giant cells, which reflect the fusion of infected microglia or macrophages. Most of the isolates had minimal if any cytopathic effect in microglia, but a few isolates induced prominent syncytia. However, syncytium formation was not always associated with isolates exhibiting high levels of replication. For example, the highly microglia-tropic isolate HIV-1_{AO-5945} produced very little cytopathic effect whereas isolate HIV-1_{AL-6244}, which also grew well in microglia, was clearly cytopathic, inducing fusion in a large proportion of the cells by day 14 of infection. In

contrast, the syncytium-inducing isolate HIV-1₃₀₂₀₇₇, which showed limited growth in microglia compared with MDM, produced large multinucleated cells scattered throughout the culture. Evaluation of cytopathic effect in the MDM cultures was more difficult because uninfected MDM tend to spontaneously form multinucleated giant cells in culture whereas uninfected microglia exhibit little or no fusogenic activity. These results suggested that high replication and cytopathicity are separate phenotypes, an observation that we then confirmed with a passaged isolate (see below).

Virus passage in microglia. Several recent studies have shown that the evolution of virus sequences within the brain differs from that in the periphery, in that the virus quasi-species in the CNS are apparently more homogeneous than that in lymphoid organs like the spleen (15, 41). This observation could reflect adaptation or selection for growth in microglia within the immunologically privileged CNS environment. To test whether this adaptation could be reproduced in the laboratory, we sequentially passaged HIV-1_{B_{ORI}} and one well-characterized molecularly cloned isolate, HIV-1_{89.6}, in microglia and monitored growth by measuring the p24^{gag} concentration. HIV-1_{B_{ORI}}, which exhibited modest replication initially, adapted for growth in the microglia, reaching peak replication by passage 8 (Fig. 3). HIV-1_{89.6} did not appear to adapt for consistently high-level growth in the microglia either before or after passaging, and, unlike the other isolates, its relative level of replication varied considerably among different donors. This finding may be due to the cloned genotype of HIV-1_{89.6}, which makes it more subject to donor variability.

To confirm that HIV-1_{B_{ORI}} had been truly adapted to microglia, we compared its growth with that of HIV-1_{B_{ORI}/14} in microglia, MDM, and PBMCs at different multiplicities of infection. HIV-1_{B_{ORI}/14} produced very high p24^{gag} concentrations compared with the unpassaged stock when an inoculum of 9 ng of p24^{gag} per 2.5×10^5 cells was used (data not shown). The difference in replication was even more pronounced when 4.5 ng of p24^{gag} was used for infection. At this lower inoculum, HIV-1_{B_{ORI}} did not infect the microglia at all (Fig. 4; compare with the results for an inoculum of 50 ng in Table 3). In addition to enhanced growth in microglia, HIV-1_{B_{ORI}/14} produced higher p24 levels in MDM than did the original stock. We also compared the growth of these two viruses in stimulated PBMCs and found that HIV-1_{B_{ORI}/14} now exhibited a diminished capacity for replication in PBMCs, which was evident when a low virus inoculum was used (Fig. 4).

HIV-1_{B_{ORI}/14} also had greater capacity to induce CPE, although its peak p24^{gag} titer did not appreciably increase following the eighth passage. Figure 5 depicts microglia infected with equivalent inocula of the HIV-1_{B_{ORI}} (Fig. 5A) and HIV-1_{B_{ORI}/14} (Fig. 5B). The multinucleated cells induced by HIV-1_{B_{ORI}/14} were similar to the characteristic giant cells observed in the brains of HIV-1-infected individuals (6).

PCR and sequence analysis. Many groups have demonstrated that the gp120 V3 loop contains elements important for cell tropism and syncytium formation (8, 38, 47). Therefore, the V3-loop sequence of HIV-1_{B_{ORI}} was determined throughout passage by PCR amplification of DNA prepared from infected microglia with nested primer sets (see Materials and Methods).

A comparison of V3 sequences of HIV-1_{B_{ORI}} at selected passages is shown in Fig. 6. In general, the sequences conform to the MDM-tropic consensus sequence (10), but by passage 8, there were a small number of changes, including the presence of a leucine residue at position 332. This amino acid change has been proposed as one potential envelope signature for sequences obtained from individuals with HIVD (41). In ad-

FIG. 5. Syncytium formation in microglia. Replicate microglial cultures were infected at 37°C for 16 h with HIV-1_{BORI} (A) or HIV-1_{BORI/14} (B) inocula containing 20 ng of p24^{gag}. The cultures were incubated for 9 days and then labeled with Di-I-Acylated LDL (5 µg/ml) and Hoechst stain (20 µg/ml) for 2 h at 37°C. The cells were washed, fixed in 4% paraformaldehyde, and photographed with an Leitz Aristoplan microscope with an epifluorescent source.

Isolate	275	295	315	335
MDM con	ENFTNNAKTIIVQLNESVEINCTRPNNNTKRSIHIGPGRAFYTTGEIIGDIRQAHNCNISRAKW			
HIV-1 _{BORI}	T.....G.....T.H...D.....			
HIV-1 _{BORI/4}	K.I.TL.VI.....T.....D.....			
HIV-1 _{BORI/8}	...S.....T.....T.....D.....Y. IL ..T..			
HIV-1 _{BORI/15}	T.....T.....D.....Y. IL ..T..			

FIG. 6. Sequence analysis of the V-3 loop region of microglia-passaged HIV-1_{BORI}. DNA from microglial cultures infected with HIV-1_{BORI}, HIV-1_{BORI/4}, HIV-1_{BORI/8}, and HIV-1_{BORI/15} were PCR amplified with a nested primer set for the V-3 loop region. PCR products were gel purified and sequences with an ABI 373A automated sequencing system. HIV_{BORI} sequences are compared with the macrophage-tropic consensus (MDM con) (9). Boldfaced residues were reported to be associated with HIVD by Power et al. (40, 41).

dition, a second potential HIVD signature, a histidine at position 308 was maintained in the genotype throughout passaging. Although HIV-1_{BORI} developed increased cytopathicity at later passages, there were no subsequent amino acid changes in this region (Fig. 6, HIV-1_{BORI/15}) which could be correlated with the change in phenotype.

DISCUSSION

Under the assumption that MDM and microglia are similar, relatively few studies have addressed the potential role of microglia tropism in HIV neuropathogenesis (40, 50). However, although MDM and microglia are derived from common precursors in the bone marrow, express similar surface markers, and perform roughly similar functions (54), they exhibit some inherent differences. While perivascular monocytes appear to traffic across the blood-brain barrier and are continually replaced, intrinsic microglia remain resident within the brain parenchyma (32). Resting microglia exhibit a characteristic ramified morphology, which is distinguishable from the amoeboid appearance of MDM. In addition, microglia and MDM show distinct differences in their membrane potassium channel patterns, with microglia displaying much greater sensitivity to depolarization (25, 26). This increased responsiveness to depolarizing signals, such as ATP release from dying cells, may potentially affect microglia function or activation in response to viral infection.

Using a panel of both laboratory and primary HIV-1 isolates, we found that, as previously reported, MDM tropism and microglia tropism are highly overlapping phenotypes (50). However, our data indicate that in the context of HIV infection, microglia are distinct from MDM. First, some T-cell-tropic viruses that normally do not replicate in MDM were capable of highly productive infection in the microglial cultures, suggesting that intrinsic differences exist between microglia and MDM. However, since high-level replication of these viruses occurred in about 50% of the cultures, it appears that cells from only a subset of donors are permissive for replication of T-cell-tropic strains.

More generally, we looked at a large group of isolates obtained during primary viremia (2, 11, 21). This initial burst of viral replication is likely to be critical for the establishment of persistent infection in many organs (22), including the CNS (20, 45). Several findings were salient. Most of the primary viruses replicated well in both cell types; in fact, microglial cultures produced higher p24^{gag} concentrations than did similarly infected MDM. Of greater potential interest was the finding that some isolates consistently exhibited preferential tropism for one of the two cell types: four of the isolates grew better in MDM than in microglia, and five isolates produced at least 10-fold more p24^{gag} in microglia than in MDM. Our results indicate that microglial tropism can be at least partially separated from tropism for MDM. Furthermore, two of the

five isolates that demonstrated preferential growth in microglia were associated with individuals who had neurological symptoms at the time of primary infection. Although more HIVD-associated isolates need to be evaluated for confirmation of this observation, these findings suggest that the ability of a virus to replicate in microglia may be associated with early clinical manifestations. More importantly, they indicate that microglia tropism can be at least partially separated from tropism for MDM.

A potential biological explanation for these results may come from the preliminary observation that while freshly isolated monocytes are resistant to HIV-1 infection, microglia can be infected as early as 1 day after isolation and, in contrast to MDM, do not require stimulation with growth factors (data not shown). This indicates that the highly differentiated phenotype of microglia makes these cells more susceptible to HIV infection, which may be an important factor in the establishment of CNS infection during the early stages of infection. Those HIV strains that replicate more robustly in microglia may be inherently more adapted to the mature cellular milieu of the CNS cells.

Several investigators have proposed that virus entering the brain is sequestered and evolves independently, possibly becoming adapted for microglial replication (15, 39, 41). Lane et al. (31) have demonstrated that in vivo passaging of a brain-derived simian immunodeficiency virus (SIV) isolate resulted in a more neuropathogenic and homogeneous virus quasi-species than the original stock. In a second series of experiments, we mimicked this adaptation in vitro. One primary isolate (HIV-1_{BORI}) was adapted to microglia by sequential passaging, with 1,000-fold greater production of p24^{gag}. In contrast HIV-1_{89.6}, a molecularly cloned MDM-tropic isolate, demonstrated no adaptation. Power et al. (41) have suggested that specific amino acid changes in the V3 loop are associated with viral persistence in the CNS and the development of HIVD, although these changes have not been found in all studies (12, 30, 44). The sequence of the late-passage HIV-1_{BORI/14} maintained the signature histidine residue at position 308, which was already present before passaging, and in addition had selected a leucine at position 332, the second important difference noted by Power et al. (41). However, it is unlikely that a single amino acid change in the V3 region is responsible for all of the phenotypic differences noted in the passaged virus, since no additional changes were noted in this region as the virus developed increased cytopathology. It is possible that changes occurred in other regions of gp120, such as V1 and V2, which reportedly are important for the spread of virus in MDM cultures, affected the phenotype of the passaged virus (48). Taken together, these results indicate that in vitro passage in microglia may imitate the selection process that has been postulated to occur in the CNS, resulting in viruses with increased replication and ability to form syncytia in cells of the CNS.

In light of the finding that most of these primary isolates could infect microglia, albeit at different levels, one still wonders whether specific viral tropisms are related to the clinical observation that only a proportion of HIV-1-infected individuals develop HIVD. One possibility is that a threshold level of replication is necessary before a persistent infection can be established in the brain. This would be consistent with data from some investigators, who have suggested that the level of HIV replication is related to the development of HIVD (13, 51, 52). However, it is also possible that neuroinvasiveness and neurovirulence are separable phenotypes and that the induction of disease is related to other properties in addition to microglial replication. In this regard, a recent study involving quantitative PCR suggested that HIV-infected individuals with and without HIVD contain similar levels of viral DNA, although RNA levels, which may better reflect virus infection, could not be quantitated (23). Further studies are necessary to delineate the relationship between HIV-1 replication in microglia and the development of HIVD, but our results provide a framework in which to investigate microglial tropism.

ACKNOWLEDGMENTS

This study was supported by PHS grants NS-27405 and NS-30606.

We thank Ron Collman (University of Pennsylvania), David Ho (Aaron Diamond AIDS Center), and George Shaw (University of Alabama) for providing isolates from acutely ill patients and Andy Pekosz, Neal Nathanson and Joseph Shieh for helpful comments. The following HIV strains were obtained from the NIH AIDS Research Reagent and Reference Program: JrCSF, JrFL, SF162, IIIB, NDK, ADA, NL4-3, YU-2, DV, and BaL.

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