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An unusual syncytia-inducing human immunodeficiency virus type 1 primary isolate from the central nervous system that is restricted to CXCR4, replicates efficiently in macrophages, and induces neuronal apoptosis

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Macrophage/microglia cells are the principal targets for human immunodeficiency virus type 1 (HIV-1) in the central nervous system (CNS). Prototype HIV-1 isolates from the CNS are macrophage (M)-tropic, non–syncytia-inducing (NSI), and use CCR5 for entry (R5 strains), but whether syncytia-inducing (SI) CXCR4-using X4 strains might play a role in macrophage/microglia infection and neuronal injury is unknown. To explore the range of features among HIV-1 primary isolates from the CNS, the authors analyzed an HIV-1 strain (TYBE) from cerebrospinal fluid of an individual with acquired immunodeficiency syndrome (AIDS) that was unusual because it was SI. Like other CNS isolates, HIV-1/TYBE replicated to high level in primary human macrophages, but, in contrast to CNS prototypes, TYBE used CXCR4 exclusively to infect macrophages. A functional TYBE *env* clone confirmed the X4 phenotype and displayed a highly charged V3 sequence typical of X4 strains. Supernatant from TYBE-infected primary human macrophages induced apoptosis of neurons. Thus, TYBE represents a novel type of CNS-derived HIV-1 isolate that is CXCR4-restricted yet replicates efficiently in macrophages and induce neuronal injury. These results demonstrate that HIV-1 variants in the CNS may possess a broader range of biological characteristics than generally appreciated, raise the possibility that X4 strains may participate in AIDS neuropathogenesis, and provide a prototype clade B HIV-1 strain that replicates efficiently in primary macrophages through the exclusive use of CXCR4 as a coreceptor. *Journal of NeuroVirology* (2003) **9,** 432–441.

Keywords: chemokine receptor; envelope; neuroAIDS; tropism

Introduction

Human immunodeficiency virus (HIV) encephalopathy (HIVE) is directly linked to virus replication within the central nervous system (CNS). Macrophage/microglia cells are the principal targets for productive infection in the CNS, and infection of these cells plays a critical, albeit still incompletely understood, role in the development of HIVE. The

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mechanisms by which macrophage/microglia infection lead to neurological dysfunction and damage are believed to involve viral gene products, particularly the envelope glycoprotein, as well as cellular soluble factors that are induced by viral infection (reviewed in Kaul *et al*, 2001). HIV-1 isolates are generally grouped into macrophage (M)-tropic, T-cell line (T)-tropic, and dual-tropic variants. M-tropic isolates replicate in primary macrophages (and microglia) and lymphocytes but not cell lines, are non–syncytia-inducing (NSI) *in vitro*, and use the chemokine receptor CCR5 as a coreceptor for entry (R5 strains). T-tropic isolates replicate in lymphocytes and CD4-positive cell lines but not primary macrophages, are syncytia-inducing (SI) *in vitro*, and use the coreceptor CXCR4 (X4 strains). Dual-tropic strains replicate in all three target cell types, form syncytia *in vitro*, and use both CCR5 and CXCR4 (R5X4 strains). Prototype HIV-1 isolates derived from the CNS all display the M-tropic, CCR5-dependent features of R5 variants, which is consistent with macrophages and microglia as the principal CNS viral reservoir (Gartner *et al*, 1986; Cheng-Mayer *et al*, 1989). As a result, this type of variant is generally considered to be exclusively responsible for infection in the CNS.

Recently, however, several observations have raised the question of whether CXCR4-using isolates may play a role in CNS infection in acquired immunodeficiency syndrome (AIDS). We and others recently showed that primary human macrophages express CXCR4 as well as CCR5, and that certain primary HIV-1 isolates are able to infect macrophages through CXCR4, even though prototype laboratory-adapted SI X4 strains cannot (Yi *et al*, 1998, 1999; Simmons *et al*, 1998; Verani *et al*, 1998). Microglia also express CXCR4 (Albright *et al*, 1999; Lavi *et al*, 1997). This indicates the possibility that variants with the potential to infect the principal CNS reservoir might be broader than generally appreciated. *In vivo*, CXCR4 is widely expressed within the CNS, both in conjunction with CD4 on macrophages and microglia, and in the absence of CD4 on neurons and other cells (Lavi *et al*, 1997; Westmoreland *et al*, 1998; Vallat *et al*, 1998). Importantly, HIV-induced neuronal injury can result from interaction between the viral envelope glycoprotein gp120 and CXCR4 on neurons or other target cells (Hesselgesser *et al*, 1998; Bezzi *et al*, 2001; Catani *et al*, 2000). Furthermore, in mixed brain cultures *in vitro*, CXCR4-using isolates may possess particular neurotoxic potential (Ohagen *et al*, 1999; Zheng *et al*, 1999a, 1999b). However, all of these observations and potential mechanisms are irrelevant if isolates that interact with CXCR4 are not found within the CNS in infected individuals.

Because of this, we sought to reexamine the issue of viral phenotype and coreceptor choice among isolates from the CNS, within the context of current understanding of macrophage entry pathways. To this end, we undertook careful analysis of an HIV-1 strain (TYBE) derived from cerebrospinal fluid (CSF) of an individual with AIDS (Yi *et al*, 1999) that differed from prototype CNS isolates in that it replicated in cell lines and was SI in culture. Strain TYBE replicated efficiently in primary macrophages, and macrophage infection resulted in the production of neurotoxic secretory products. However, TYBE utilized CXCR4 exclusively as a coreceptor for infection. Thus, HIV-1 variants in the CNS may have a broader range of biological and genetic features than generally recognized. These results add credibility to the possibility that X4 strains may participate in infection and disease pathogenesis related to the CNS.

Results

Characterization of an SI primary HIV-1 isolate from the CNS

We previously generated a panel of primary isolates from the CSF of infected patients with neurological symptoms undergoing lumbar puncture for diagnostic purposes. Of eight primary isolates from CSF that were studied, one obtained from a man with neurological symptoms and designated HIV-1/TYBE (Yi *et al*, 1999) showed an unexpected phenotype in preliminary analysis. In contrast to prototype CNS strains, TYBE was SI in culture. To ensure that we were dealing with a single viral species rather than a mixture of variants, the TYBE primary isolate was subjected to biological cloning by limiting dilution on primary human peripheral blood mononuclear cells (PBMCs). Five biological clones of TYBE were analyzed and all five exhibited the same SI phenotype (data not shown), so one clone was selected at random for further study.

We tested the ability of the TYBE isolate to replicate in primary macrophages, primary lymphocytes, and the MT-2 cell line. In parallel, we utilized the prototype CNS-derived R5 M-tropic isolate JRFL, the X4 T-tropic strain 3B, and the R5X4 dual-tropic isolate 89.6. As shown in Figure 1, HIV-1/TYBE replicated in monocyte-derived macrophages (MDMs), MT-2 cells, and PBMCs. This pattern was similar to that seen with the dual-tropic prototype 89.6, which uses both CCR5 and CXCR4 for entry. However, TYBE differed markedly from the CNS prototype JRFL, which replicated in MDMs and PBMCs but not in MT-2 cells, as expected given the fact that JRFL is restricted to CCR5 use. TYBE also differed from the CXCR4-restricted prototype 3B, which replicates in PBMCs and cell lines but is unable to utilize CXCR4 on macrophages. Thus, TYBE represents an unusual type of CNS-derived HIV-1 variant.

CNS-derived syncytia-inducing strain TYBE uses CXCR4 for infection of macrophages

Because of the unexpected cell-line tropism and SI phenotype of strain TYBE, we tested whether it utilized the traditional macrophage entry coreceptor CCR5, or whether it could use macrophage

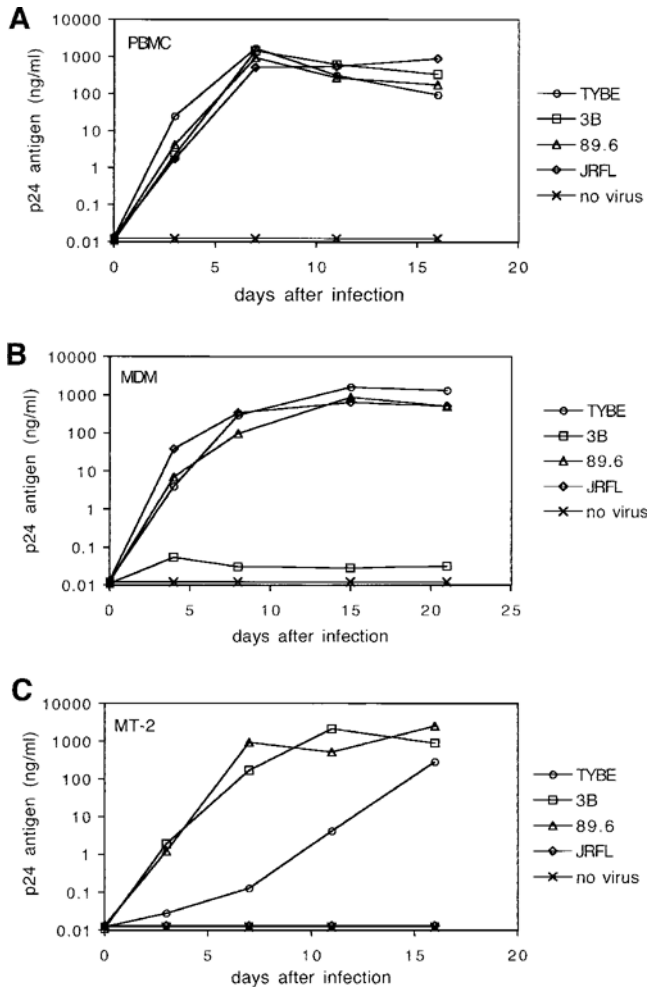


Figure 1 Replication of HIV-1 primary isolate TYBE in primary and transformed cells. Peripheral blood mononuclear cells (PBMCs) were stimulated with PHA followed by IL-2, and monocyte-derived macrophages (MDMs) were maintained in culture for 1 week prior to infection. PBMCs (**A**), MDMs (**B**), and the MT-2 cell line (**C**) were infected overnight with strain TYBE or with prototype HIV-1 strains JRFL (M-tropic), 3B (T-tropic), or 89.6 (dual-tropic) using 10 ng of p24 antigen of each virus. Cultures were sampled periodically for p24 antigen in the supernatant. Data shown are representative of three independent experiments.

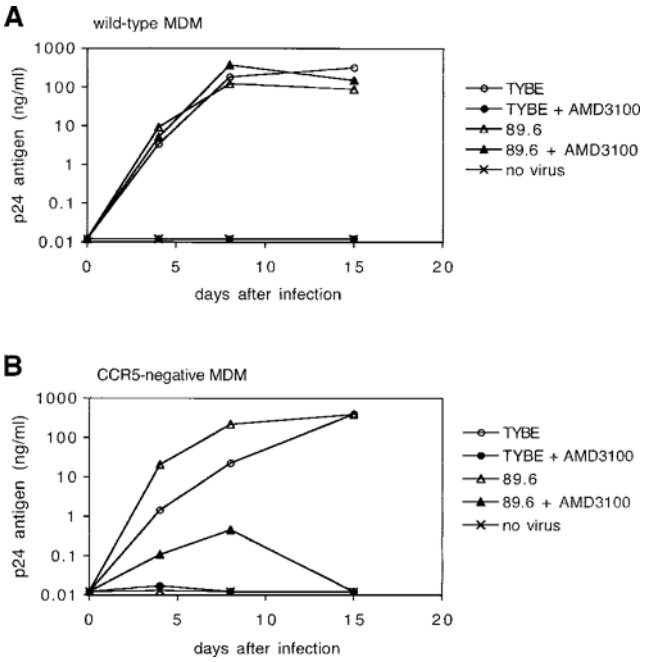


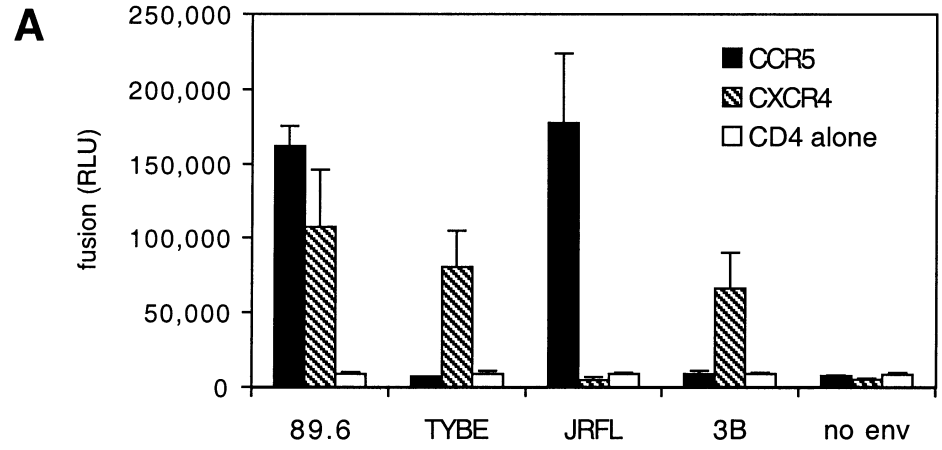
Figure 2 Coreceptor-dependent infection of macrophages by strain TYBE. MDMs from a donor homozygous for the CCR5 wild-type allele (**A**) and a donor homozygous for the CCR5 Δ32 deletion allele (**B**) were infected overnight with strain TYBE or with the R5X4 isolate 89.6 using 10 ng of p24 antigen of each virus. Infections were carried out in the presence or absence of the CXCR4 blocker AMD3100 (1 μg/ml). Supernatant was sampled periodically for p24 antigen production. Data shown are representative of three independent experiments using different cell donors.

CXCR4, as has been recently described for certain other HIV-1 primary isolates. To do this, we employed macrophages lacking functional CCR5 obtained from donors homozygous for the CCR5 Δ32 allele (Rana *et al*, 1997), in conjunction with the CXCR4-specific antagonist AMD3100 (Donzella *et al*, 1998).

As shown in Figure 2, TYBE replicated efficiently in both wild-type and CCR5-deficient MDMs. In this way, TYBE resembled 89.6, which can utilize both CCR5 and CXCR4 on macrophages for entry. However, the CXCR4 antagonist AMD3100 blocked TYBE infection of MDMs whether or not CCR5 was present. This result contrasted with 89.6, which was blocked by CXCR4 inhibition only if CCR5 was absent (Figure 2B), but not if a CCR5-mediated pathway was available (Figure 2A). As expected, the CNS-derived prototypes JRFL and YU2, as well as other R5 prototype strains, were unable to replicate in MDMs lacking CCR5 (data not shown and Rana *et al*, 1997). These data indicate that TYBE uses CXCR4 for efficient infection of primary macrophages, and does not utilize the classical CCR5 entry pathway. Because these studies employed a biological clone of TYBE, the dual macrophage and MT-2–tropic phenotype exhibited did not result from a mix of distinct SI/T-tropic and NSI/M-tropic variants within a primary isolate swarm. Thus, TYBE efficiently infects primary human macrophages through a pathway that differs from both prototype CNS-derived virus isolates and from standard dual-tropic strains.

Functional and genetic analysis of the TYBE Env glycoprotein

Because SI strains from the CNS have not previously been studied in detail, we generated a functional molecular clone of the TYBE *env* gene in order to analyze its biological and genetic characteristics. Clones were screened for orientation by restriction analysis and for function based on the ability to mediate cell-cell fusion. As shown in Figure 3**A**, the TYBE Env used CXCR4 for fusion but did not utilize CCR5. In contrast, JRFL used CCR5 only, 3B used CXCR4 only, and 89.6 used both coreceptors. Thus, the TYBE coreceptor fusion profile in transfected



B

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          [------------------V3----------------]
                                                    charge     co-R       tropism
cons      CTRPNNNTR•KSIHI••GPGRAFYTTGEIIGDIRQAHC    +3         -          -
JRFL      ---------•-----••--------------------     +3         R5         M
JCSF      ----S----•-----••--------------------     +3         R5         M
89.6      ---------•RRLS-••-------ARRN----------    +7         R5X4       dual
TYBE      ---VSK-I-Q-R-K-••------VA--•D-----K---    +7         X4         dual
3B        ---------•-R-R-QR------V-I-•K--NM-----    +9         X4         T

          [-------------V1-------------|------------------V2------------------]

JRFL      CKDV••••NATNTTNDS•EGTMERGEIKNCSFNITTSIRDEVQKEYALFYKLDVVPIDNTNNTKYRLISC
JRCSF     ----••••------SS-•--M-------------K----K-----------------K----------
89.6      -TNLNITK-T--P-SS-•W-M--K--------Y------NK-K------NR------E-•---S------
TYBE      -T-F••••-T-RANSS-•-NK--I--------K-A-N---KT--Q----N----A-----••R-S-V----
3B        -T-LK•••-D---NSS-GRMI--K----------ST---GK------F-----II-----•DTTS-K-T--
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Figure 3 Fusion function and sequence analysis of the TYBE envelope. (**A**) Cell-cell fusion mediated by Env glycoproteins from TYBE, JRFL, 3B, and 89.6. Effector 293T were infected with a T7 polymerase–expressing recombinant vaccinia virus and then transfected with plasmids encoding *env* under control of T7 promoter. Target QT6 cells were cotransfected with plasmids encoding CD4 plus CCR5 or CXCR4, and a reporter plasmid containing a T7-driven luciferase gene. Cells were mixed and luciferase levels (RLU) measured in cell lysates 6 h later as an indication of cell-cell fusion. Data shown are representative of three independent experiments. (**B**) The predicted amino acid sequence of TYBE Env regions compared with four prototype HIV-1 strains. Dashes indicate identical amino acids compared with the query sequence and dots indicate gaps. Coreceptor use is based on fusion and infection of transfected and primary cells, and tropism is based on the ability to infect primary lymphocytes and macrophages (M-tropic); primary lymphocytes and lymphoid cell lines (T-tropic); or all three target cells (dual-tropic). V3 sequences are compared with the clade B consensus whereas V1/V2 sequences are compared with each other.

cells was consistent with coreceptor usage for primary macrophage infection, and confirms that this CNS-derived SI strain is restricted to CXCR4. In addition, it indicates that its dual-tropic phenotype (i.e., replication in both cell lines and MDMs in addition to PBMCs) results from its ability to utilize CXCR4 efficiently on macrophages and cell lines (dual-tropic X4), and not from an ability to use both CCR5 and CXCR4 on macrophages and cell lines, respectively (dual-tropic R5X4) (Yi *et al*, 1999).

In addition to CCR5 and CXCR4, a variety of other chemokine and orphan receptors support HIV-1 entry or fusion *in vitro*. Therefore, we tested whether TYBE used the alternative coreceptor CCR2b, CCR3, and APJ. Of note, the latter two molecules are highly expressed in the CNS and have been proposed as contributing to pathogenesis in HIVE (He *et al*, 1997; Edinger *et al*, 1998; Choe *et al*, 2000). TYBE failed to fuse with cells expressing either CCR2b or CCR3 in conjunction with CD4, whereas APJ enhanced fusion by only twofold over cells expressing CD4 alone (data not shown). Thus, we found no evidence to support a role for efficient use of alternative coreceptors by this X4 primary isolate from the CNS, even though CXCR4 utilization is frequently associated with broader chemokine receptor utilization (Connor *et al*, 1997).

Full-length sequencing of the TYBE *env* (GenBank accession number A4189526) revealed broad conservation of critical structures (Rizzuto *et al*, 1998),

including all 19 cysteine residues in gp120, 17 out of 18 residues identified as important for coreceptor binding, and a predicted glycosylation pattern generally concordant with other HIV-1 isolates (data not shown). We then focused on the V3 region, which plays a central role in tropism, coreceptor choice, SI capacity, and other important features. As shown in Figure 3**B**, the TYBE V3 domain exhibits a +7 strong positive charge, similar to the R5X4 isolate 89.6 and markedly higher than JRFL, YU2, and other NSI/M-tropic R5 strains. Thus, the V3 region of TYBE displays a pattern typical of CXCR4-utilizing SI strains, and does not demonstrate similarity to typical CNS-derived NSI R5 isolates. Several groups have suggested that the V1/V2 domains contain important determinants of tropism, SI capacity, or pathogenesis. In particular, an extended V2 hypervariable region has been linked to the transition from NSI to SI (and presumably X4 use) (Groenink *et al*, 1993), although conversely others have correlated the extended V2 with slow disease progression and persistent CCR5 use (Masciotra *et al*, 2002). Strain TYBE exhibited a relatively short V2 hypervariable region (Figure 3**B**) and also failed to display an additional 3′ glycosylation sites that was previously linked to the SI phenotype (Groenink *et al*, 1993).

HIV-1 TYBE induction of neuronal apoptosis

The mechanisms by which productive HIV-1 infection of macrophage/microglia cells in the CNS leads to neuronal injury remain incompletely understood. Proposed pathways include both direct mechanisms involving viral proteins, particularly gp120, and indirect mechanisms whereby infected macrophages release cellular products that injure neurons. Several groups have shown that HIV-1–infected primary human macrophages produce soluble factors that injure neurons (Giulian *et al*, 1990; Xiong *et al*, 1999). Typical CCR5-dependent M-tropic isolates have been used in the great majority of these studies, and although some have suggested that X4 HIV-1 strains also have the capacity to induce neurotoxic macrophage products (Ohagen *et al*, 1999), the ability of X4 primary isolates from the CNS to induce this damage has not been tested. Therefore, we sought to determine whether HIV-1 TYBE-infected macrophage supernatant contains products that cause neuronal apoptosis. To assess neuronal injury, we utilized the NTera 2/c1.D1 human teratocarcinoma cell line (NT−), which was induced to neuronal differentiation (NT2.N neurons) and cultured with a feeder layer of rodent astrocytes. Neuronal cultures were exposed for 2 days to supernatants of TYBE-infected or uninfected MDMs, and then analyzed for apoptosis on the basis of DNA fragmentation as detected by deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) labeling (Chen *et al*, 2002).

As shown in Figure 4, few NT2.N neurons exposed to supernatant from uninfected macrophages were positive by TUNEL staining. This low level of apoptosis was not different from control NT2.N neurons that were not exposed to macrophage supernatant (data not shown). In contrast, exposure to conditioned media from MDMs infected with HIV-1 TYBE increased the proportion of neurons undergoing apoptosis by approximately sixfold. The increased apoptosis could not be accounted for by confounding factors such as mycoplasma contamination, which was absent from all supernatants as determined by polymerase chain reaction (PCR) (data not shown). This experiment was repeated four times using supernatants from MDMs derived from different blood donors, and similar results were seen. Although the absolute levels of apoptosis in NT2.N varied among experiments, in all cases there was a marked and statistically significant increase in neuronal apoptosis induced by TYBE-infected macrophage supernatants. If macrophages were exposed to virus in the presence of AZT to block infection, the supernatant exhibited no neurotoxicity (data not shown), indicating that macrophage infection, and not merely exposure to virus or cellular supernatant, was required. Also, in this model, apoptosis is prevented if neuronal N-methyl-D-aspartate (NMDA) receptors are blocked, but not if neuronal chemokine receptors are blocked (Chen *et al*, 2002), indicating that macrophage involvement is required and it is not direct NT2.N exposure to virus or gp120 that causes the neuronal injury.

Discussion

The role of chemokine receptors in the pathogenesis of HIV-associated neurological disease is an area of considerable importance but considerable confusion as well. This uncertainty stems at least in part from the dichotomy between the longstanding observation on one hand that M-tropic NSI variants (subsequently shown to use CCR5 and not CXCR4 for entry) are the predominant viral species found in the CNS and, on the other hand, findings that CXCR4 is widely expressed in the CNS *in vivo* and can mediate neurological injury in several important models *in vitro* (Hesselgesser *et al*, 1998; Meucci *et al*, 1998; Zheng *et al*, 1999a, 1999b). Our study addresses one facet of this dilemma, by confirming the existence of X4 HIV-1 in the CNS, and providing careful biological and genetic characterization of this type of isolate.

Although our study is the first to clearly identify and analyze an X4 CNS-derived HIV-1 primary isolate, a critical question is whether this represents a rare or perhaps singular situation, or whether X4-using CNS isolates may have general relevance in HIV-1 infection. Of note, although established dogma links exclusive CCR5 use with CNS virus isolates, certain previously published data suggest that TYBE may not be an entirely unique variant. One of the earliest CNS-derived HIV-1 isolates, HIV-1/BR (Anand *et al*, 1989), was isolated from brain of an individual

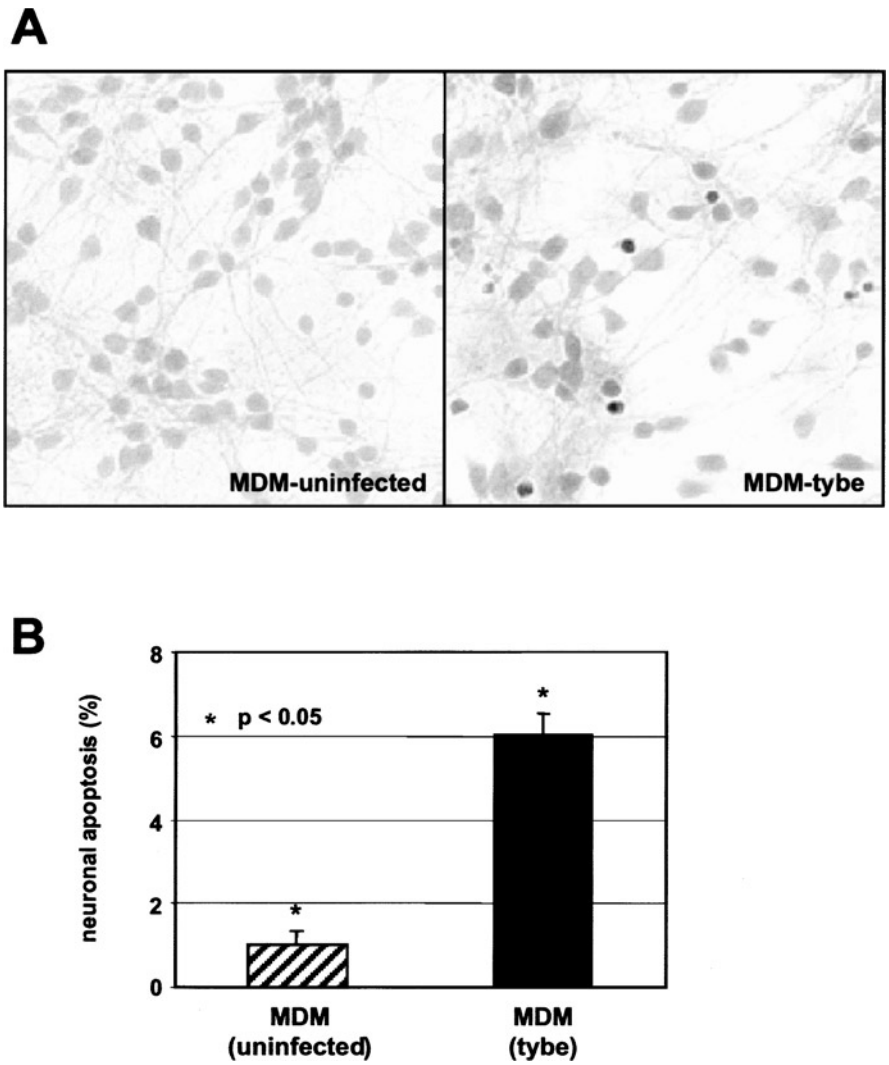

Figure 4 Neuronal apoptosis in NT2.N/astrocyte cocultures induced by HIV-1 TYBE-infected MDMs. NT2.N neurons were cultured on glass coverslips with a feeder layer of rodent astrocytes for 5 weeks, exposed for 48 h to supernatants from HIV-1 TYBE-infected or uninfected MDMs, and then examined by TUNEL assay and Hoechst 33342 staining as described in the text. (**A**) Phase-contrast photographs of TUNEL images from representative fields of neuronal/astrocyte cultures after exposure to MDM supernatants. Cells with dark staining are TUNEL-positive neurons. Magnification 200×. (**B**) Percentage of neurons positive for apoptosis by TUNEL stain. The data represent the mean ±SEM of two coverslips per treatment condition from one representative experiment. The experiment was repeated four times using MDMs from different donors, with similar results. Comparisons of means were made by Student's *t* test.

with severe neurological disease and was described as highly cytopathic and SI, with a strong positive V3 charge. Although coreceptor usage for this isolate has not been defined, the SI phenotype and V3 sequence suggests that it is likely X4 or R5X4. SI isolates from the CSF have been described (Brew *et al*, 1996), and in a recent report, two of six primary isolates derived from brain tissue were found to utilize CXCR4 as a coreceptor (Gorry *et al*, 2001). Our data together with those results argue that the biological and coreceptor features of HIV-1 species in the CNS are worthy of reevaluation and careful analysis. Further study of CNS viral variants will be required to define the potential role played by X4-using species in the pathogenesis of HIVE. Importantly, it is possible that critical pathogenic roles may be played by minority variants present either in low abundance relative to more easily sampled species in the CNS or sequestered in a regional or cell-specific manner.

One limitation of our study is that the subject from whom this strain was isolated did not have clearly defined HIVE, as later in the course of his illness evidence of cytomegalovirus (CMV) infection was identified. Whether the HIV-associated brain injury of HIVE may have been associated with this variant is unknown, because pathological tissue was not available and clinical evidence would be obscured by possible CMV coinfection. On the other hand, important biological and genetic information about CNS viral variants has been derived from similar subjects in

the past, including the HIV-1 strains JRFL and JRCSF, which also were derived from an individual with CMV encephalitis (Koyanagi *et al*, 1987; Pang *et al*, 1991). Thus, although strain TYBE may serve as a prototype of a CNS-derived CXCR4-restricted, highly M-tropic variant, a clear-cut link to *in vivo* pathogenesis remains to be fully defined. Another limitation of our study is that this isolate was obtained from the CSF rather than primary brain tissue. Although the origin of virus in the CSF remains uncertain, some authors have suggested that early in disease it arises from the extraneural sources, whereas later in disease it originates from intraparenchymal sources in the CNS compartment (Haas *et al*, 2000; Ellis *et al*, 2000). The patient from whom TYBE was isolated had advanced AIDS, but the unavailability of primary brain tissue precludes direct comparison of this isolate with variants that may be present within the brain itself.

Neurological complications of AIDS occur most frequently in the late stages of infection. It has generally been assumed that the higher prevalence with advanced disease reflects either the development of progressive immune depletion, or a time-dependent accumulation of intra-CNS infection. However, late-stage disease is also the time when CXCR4-using variants tend to emerge in the systemic compartment. Our results, together with those of others describing CXCR4-linked *in vitro* mechanisms of neuronal injury (Hesselgesser *et al*, 1998; Ohagen *et al*, 1999; Zheng *et al*, 1999a, 1999b), raise the question of whether CXCR4 use per se may have a contributory role in the late-stage prevalence of neurological complications. It would be useful to determine whether the emergence of X4 variants (in extraneural sites) is associated with a greater propensity to develop neurological disease when controlled for other parameters of immune deficiency or viral load. Of note, our study cannot answer the question of whether selective utilization of CXCR4 versus CCR5 for macrophage infection affects the level of neurotoxicity induced, because most strains we have tested, including R5 and R5X4 variants, induced neuronal apoptosis, and TYBE was within the general range seen among all isolates (data not shown).

In conclusion, we present the first well-characterized CXCR4-restricted SI HIV-1 primary isolate from the CNS. Our results demonstrate that such a phenotype is compatible with CNS infection and/or invasion, and suggest that the ability to replicate efficiently in macrophages is a necessary feature for CNS infection regardless of coreceptor usage. Combined with CXCR4-mediated mechanisms of HIV-1 neuronal injury identified *in vitro*, our data raise the possibility that X4 viruses may contribute to neurological disease in AIDS. In addition, this strain may serve as a useful prototype among clade B strains for a primary isolate with a CXCR4-restricted highly M-tropic phenotype.

Materials and methods

Virus isolation

CSF was obtained by lumbar puncture, clarified by centrifugation, and virus was isolated from cell-free fluid by coculture with PBMCs using standard methods. Coculture supernatant was tested periodically for viral p24 antigen by enzyme-linked immunosorbent assay (ELISA) (Coulter, Hialeah, FL), and when a positive value was detected, the virus was amplified in PBMCs, clarified by centrifugation, quantified by p24 antigen content, and stored at -80°C. Biological cloning was carried out by limiting dilution on primary human PBMCs. Of note, in a previous publication (Yi *et al*, 1999), the TYBE primary isolate swarm was incorrectly described as derived from the CSF cell pellet rather from cell-free CSF.

Cells and infections

Blood donors were screened for the CCR5 Δ32 deletion allele by PCR as described (Rana *et al*, 1997) and only donors homozygous for the wild-type allele were used, unless otherwise specified. PBMCs were isolated from heparinized blood by Ficol-Hypaque separation. Monocytes were purified from PBMCs by a stringent two-step selective adherence procedure as described (Collman *et al*, 1989), plated at 2×10^5 cells per well in 48-well plates and cultured for 1 week to allow differentiation into MDMs prior to infection. PBMCs were depleted of monocytes and stimulated with phytohemagglutinin (PHA) for 3 days prior to infection, and then maintained with interleukin (IL)-2. MDMs, PBMCs, and MT2 cells were infected using 10 ng of p24 antigen of each virus, washed extensively, and supernatant was sampled periodically for p24 antigen release. To define the role of CCR5 in infection, we used macrophages from donors homozygous for the defective CCR5 Δ32 allele, and to test the role of CXCR4, we used the specific antagonist AMD3100 (Donzella *et al*, 1998).

Envelope cloning

PBMCs were infected with HIV-1 TYBE and lysed 3 days later (100 mM KCl, 20 mM Tris pH 8.0, 0.1% NP-40, 0.5 mg/ml proteinase K) to obtain total cellular DNA. DNA-containing lysate from 2×10^5 cells was subject to PCR amplification using *env* amplification primers as described previously (Singh *et al*, 1999) and rTth polymerase (Perkin-Elmer, Foster City, CA) for enhanced fidelity. Amplification used an initial incubation at 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, 54°C for 5 min, and 72°C for 7 min, and a final extension at 72°C for 10 min. PCR products were then cloned into the vector PCR-blunt (Invitrogen, Carlsbad, CA), which contains the T7 promoter upstream of the cloning site. Clones were screened for orientation by restriction analysis and for function based on the ability to mediate

cell-cell fusion. Fusion was tested using a standard recombinant vaccinia virus–based cell-cell fusion assay in which Env is expressed under control of the T7 promoter, and each coreceptor is expressed in conjunction with CD4 in target QT6 quail cells (Singh *et al*, 1999).

Neuronal apoptosis assay

Neuronal injury was examined using the NTera 2/c1.D1 human teratocarcinoma cell line (NT−), which was induced to neuronal differentiation (NT2.N neurons) as previously described (Pleasure *et al*, 1992). Mature NT2.N neurons were cultured on glass coverslips at a density of 2×10^5 cells/cm^2 with a feeder layer of rodent astrocytes. The astrocyte feeder layer (5×10^4 cells/cm^2) was prepared from rat embryos as previously described (Llanes *et al*, 1995) in accordance to standard protocols, compliant with National Institutes of Health (NIH) guidelines and approved by the University of Pennsylvania Institutional Review Board. Neuronal cultures were maintained for 5 to 7 weeks to ensure full expression of NMDA and non-NMDA type glutamate receptors (Younkin *et al*, 1993) and then exposed to macrophage supernatants at a final dilution of 1:4 (125 μl of supernatant in 500 μl total volume per well). Forty-eight hours later, the cultures were fixed in 3.7% formaldehyde and subjected to terminal TUNEL with a TdT-FragEL DNA fragmentation detection kit (Oncogene Research Products, Cambridge, MA) according to the manufacturer's protocol, using a diaminobenzidine (DAB) substrate. Coverslips were counterstained with Hoechst 33342 (Calbiochem, San Diego, CA) to assess nuclear morphology. Five fields surrounding the center point of the coverslip were selected at random and digitally photographed under brightfield microscopy on an Olympus IX70 inverted scope with a Hamamatsu Color-Chilled 3 CCD camera (Hamamatsu Phototonics K.K. Japan). TUNEL-positive and TUNEL-negative neurons were counted by blind examiner utilizing Scion Image software (version 1.62c; Scion, Frederick, MD). A minimum of 1000 neurons were scored on each coverslip and two to three independently treated coverslips were scored for each treatment condition.

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