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Infection of Brain Microglial Cells by Human Immunodeficiency Virus Type 1 Is CD4 Dependent

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In the central nervous system of AIDS patients, human immunodeficiency virus (HIV) infects primarily microglia, a cell type of bone marrow origin. Moreover, microglial cells isolated from adult human brain support the replication of macrophage-adapted strains of HIV type 1 (HIV-1) (B. A. Watkins, H. H. Dorn, W. B. Kelly, R. C. Armstrong, B. Potts, F. Michaels, C. V. Kufta, and M. Dubois-Dalcq, Science 249:549–553, 1990). To determine whether the CD4 receptor, which is expressed in brain, mediates the entry of HIV-1 in microglial cells, we analyzed CD4 transcript expression in cultured microglia using highly sensitive polymerase chain reaction detection of cDNAs synthesized from RNA. With this method, CD4 transcripts could be detected in cultured microglia—as well as in various human brain regions and cultured macrophages used as positive controls—along with transcripts for the LDL and Fc receptors which are characteristic of cells of the macrophage lineage. We then attempted to block viral entry into microglial cells using anti-CD4 antibodies or soluble CD4 (sCD4), which recognize binding sites on CD4 and HIV-1 glycoprotein gp120, respectively. Cultures were pretreated with blocking antibodies (Leu-3a, OKT4A) or virus was preincubated with sCD4 prior to infection with HIV-1 strain AD87(M) or BaL. With either viral strain, these treatments resulted in the prevention of infection or significant and dose-dependent reduction in the number of infected cells and in the levels of reverse transcriptase or p24 antigen released in the medium. Thus, brain-derived microglial cells, which are the primary target of HIV-1 infection in the brain, express the CD4 receptor and this receptor is effectively used for viral entry in vitro.

Human immunodeficiency virus type 1 (HIV-1) can replicate in the central nervous systems (CNS) of AIDS patients, causing neurologic dysfunction, encephalitis, and/or myelopathy. Virus is expressed mostly in microglial and multinucleated giant cells in the patient's brain (1, 10, 11, 16, 34). Similarly, infection of primary cultures derived from adult human brain with macrophage-adapted strains of HIV-1 resulted in selective replication in microglial cells (35). These cells are members of the monocyte/macrophage lineage and are thought to migrate from the bone marrow to the nervous system early during development (26). HIV-1 infection of cultured microglial cells caused their progressive clustering, fusion, and cell death, reminiscent of the microglial nodules and multinucleated giant cells seen in the brains of AIDS patients (1, 16, 33, 34).

The CD4 molecule which functions as the HIV-1 receptor in T lymphocytes and monocyte/macrophages (8, 21, 31) is also expressed in brain, but the role of this receptor in HIV-1 entry into nerve cells has been questioned (4, 7, 12, 36). Thus, investigating the mechanism of HIV-1 entry into microglial cells may elucidate the mechanism of virus spread into the brain. In the present study, we examined the expression of CD4 transcripts in primary human brain cultures enriched in microglial cells. The role of this surface receptor in the establishment of HIV-1 infection of microglial cells was evaluated with anti-CD4 monoclonal antibodies binding to the HIV-1 receptor site on the CD4 molecule (30) as well as with soluble CD4 (sCD4) (7). Our results suggest that CD4-mediated viral entry is a major factor in the spread of HIV-1 to the CNS.

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MATERIALS AND METHODS

Tissues and cells. Regions of adult human brain tissue were obtained from a 31-year-old man at autopsy (courtesy of J. Orenstein, George Washington University) and frozen on dry ice until RNA could be extracted. Temporal lobe biopsy specimens (resected from patients with intractable epilepsy) and malignant glioma tissues were enzymatically dissociated and cultured in Dulbecco minimum essential medium supplemented with 5% fetal bovine serum and 10% giant cell tumor (GCT) supernatant (35). These cultures were used for HIV-1 infection or for RNA extraction and analysis. Enriched monocyte/macrophage cells were prepared from human peripheral blood (courtesy of F. Michaels, National Cancer Institute). The A3.01 cell line was maintained in RPMI medium supplemented with 10% fetal bovine serum.

Preparation of RNA. Total RNA was prepared by guanidinium isothiocyanate-CsCl extraction (22). Poly(A)$^+$ RNA was enriched from the autopsy tissue and A3.01 cell RNAs by oligo(dT) chromatography. Analysis of the cultured monocyte/macrophage and brain cell cultures was performed on total RNA. Extraction of RNA from the small number of microglial cells required the addition of 20 μg of *Escherichia coli* rRNA (Boehringer Mannheim) as the carrier; also, the guanidinium isothiocyanate-CsCl gradients were miniaturized and centrifuged in a Beckman airfuge for 7 h at 20 lb/in^2 (29).

RNA analysis by PCR. RNA (1 to 10 μg) was reverse transcribed (Moloney murine leukemia virus reverse transcriptase [RT]; Stratagene), and one-fourth of the cDNA was amplified for 40 cycles in a Perkin-Elmer Cetus thermal cycler (94°C for 1 min, 60°C for 2 min, 70°C for 3 min; *Taq* DNA polymerase; Stratagene) as described by Kawasaki

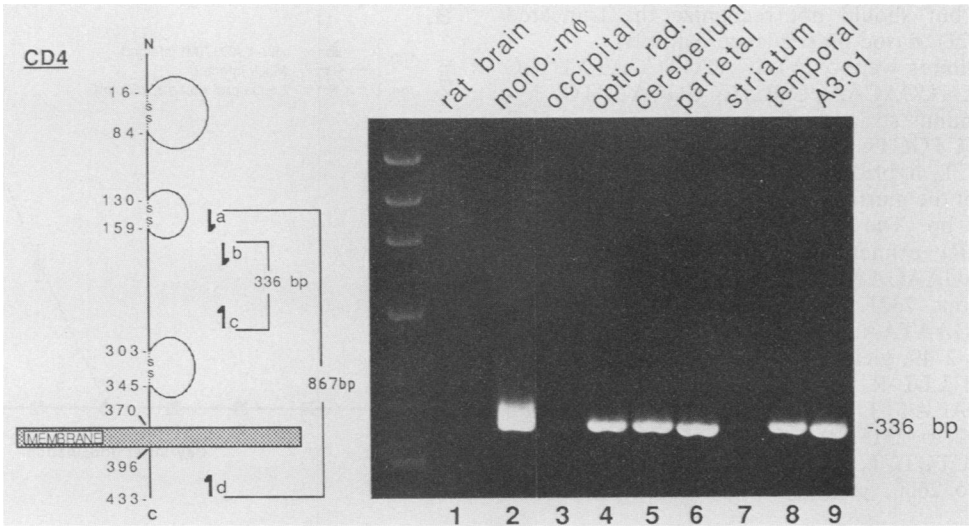


FIG. 1. PCR detection of CD4 transcripts in brain tissue and lymphoid cells. CD4 transcript expression was assayed by PCR amplification of cDNAs with sets of nested primers. The cDNAs were prepared from fresh brain tissue (lanes 1 and 8), postmortem human brain tissue (lanes 3 to 7), or cultured human cells (lanes 2 and 9) (lane 1, whole rat brain; lane 2, enriched peripheral blood monocyte/macrophages; lane 3, occipital cortex; lane 4, optic radiation; lane 5, cerebellum; lane 6, parietal cortex; lane 7, striatum; lane 8, human temporal lobe biopsy specimen; lane 9, A3.01 T lymphocytes). The position of the primers is schematized in the diagram, and the predicted fragment size was 336 bp. These primers react specifically with the human but not the rat CD4 sequences (lane 1). CD4 transcript expression in these six autopsy specimens was verified with the external primers and by Northern (RNA) analysis. All samples contained β-actin transcripts (data not shown). mφ, Macrophage.

and Wang (14). A 1-μl sample of the initial polymerase chain reaction (PCR) amplification with the outside primer pair was amplified in a sequential reaction with a primer pair nested inside the first primer pair. The CD4 primers are schematized in Fig. 1. The outside primers were 5′-GTGTC TCAGCTGGAGCTCCAGGATAGTGGC, hybridizing to the minus strand at positions 511 to 540, and 5′-TCA AATGGGGCTACATGTCTT, hybridizing to the plus strand at positions 1357 to 1377, yielding an expected fragment of 867 bp. The inside CD4 primers were 5′-GTCTTGCA GAACCAGAAGAAGGTGGAGTTCAAAATAGACATCG TGGTG, hybridizing to the minus strand at positions 556 to 603, and 5′-CGCTTTTGTCCTTTCAACGTAGTCCTTCA CTTGGACCACCACTACTCT, hybridizing to the plus strand at positions 907 to 954, yielding an expected fragment of 336 bp. When the inside primers were tested directly on unamplified A3.01 cDNA, the same 336-bp band was observed. These CD4-specific primers recognize the 3.0-kb

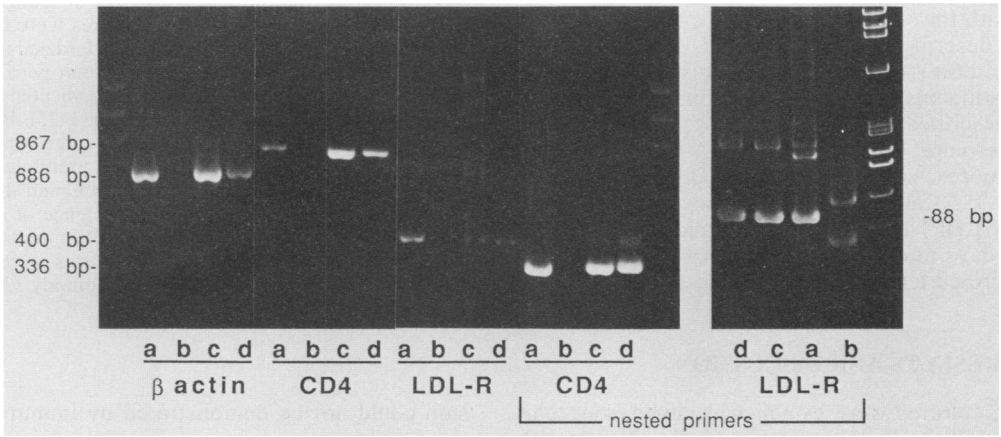


FIG. 2. CD4 transcripts are detected in enriched human microglial cultures (35). Transcript expression was assayed by PCR amplification of cDNAs prepared from enriched cultures of human peripheral monocyte/macrophages (lanes a), normal human temporal lobe cultures containing ~40% microglial cells (lanes c), or malignant glioma cultures containing >99% microglial cells (lanes d). *E. coli* rRNA (lanes b) was used as a negative control and processed through the cDNA and PCR steps since it had been added to the microglial cultures as a carrier during the RNA purification. Predicted fragment sizes are as follows: β-actin, 686 bp; CD4, 867 bp with external primers, 336 bp with nested internal primers; LDL-R, 400 bp with external primers, 88 bp with nested internal primers. Similar results were obtained from a total of four cultures derived from three different patients. Samples were processed and assayed as described in the legend to Fig. 1 except that only total RNA was used and the small number of microglial cells required the addition of *E. coli* rRNA as a carrier.

mRNA species but should not recognize the truncated mRNA species (21), based on sequence data (9a).

The β-actin primers were 5′-ATGGATGACGATATCGC TGCGCTGGTCGTCGACAACGGCTCCGGCATGTG, hybridizing to the minus strand at positions 1 to 50, and 5′-G TGGCCATCTCCTGCTCGAAGTCTAGAGCAACATAG CACAGCTTCTCT, hybridizing to the plus strand at positions 639 to 687 of the murine sequence, yielding a predicted fragment of 687 bp. The outside low-density lipoprotein receptor (LDL-R) primers were 5′-ACGTTGCTGGCA GAGGAAATGAGAAGAAGC, hybridizing to the minus strand at positions 2321 to 2350, and 5′-CCTGTTCTG CCTCCCAGATGAATAA, hybridizing to the plus strand at positions 2724 to 2749, yielding a predicted fragment of 400 bp. The internal LDL-R primers were 5′-ACCACAGAG GATGAGGTCCACATTT, hybridizing to the minus strand at positions 2491 to 2315, and 5′-CACGTCATCCTCCA-GACTGACCATCTGTCT, hybridizing to the plus strand at positions 2577 to 2606, yielding a predicted fragment of 88 bp.

Most PCR samples were analyzed on agarose gels with ethidium bromide staining and compared to ϕX174 replicative form DNA-*Hae*III size markers (New England Bio Labs). The LDL-R fragments were resolved on 4 to 20% TBE (Tris-borate-EDTA) gels (Novex).

Viruses and infection procedures. The AD87(M) and BaL strains of HIV-1 were previously shown to replicate in microglia of these human brain cultures (35). For all blocking studies, a standardized inoculum of 1×10^4 to 2×10^4 cpm of RT activity per 35-mm petri dish was used. Cultures were pretreated with or without anti-CD4 monoclonal antibody (0.39 to 25 μg/ml; Leu-3a from Becton Dickinson; OKT4 or OKT4A from Ortho Diagnostic Systems) for 1 h at 4°C and then incubated with HIV-1 for 1 h at 4°C in the continued presence of antibody. Cultures were then washed three times in Dulbecco modified Eagle medium (4°C) and refed with medium. OKT4 antibody was included as a control since it does not inhibit HIV-1 gp120 binding. Control infected and mock-infected cultures were treated identically except that the antibody or virus was omitted.

The recombinant sCD4 (supplied by R. W. Sweet, Smith Kline & French Laboratories) blocking was performed by mixing sCD4 with the virus for 1 h at 4°C prior to infecting cells for 1 h as described above.

Supernatant culture samples were harvested at the indicated times postinoculation and assayed for RT and $p24^{gag}$ as previously described (35).

Immunofluorescence. OKT4- and OKT4A-treated cultures were double fluorescently stained with DiI-LDL (1,1′-dioctadecyl-1-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate-conjugated LDL) to reveal LDL-R and HIV-1 $p17^{gag}$ antibody at 20 days after inoculation with HIV-1_{BaL}, using previously described techniques (35).

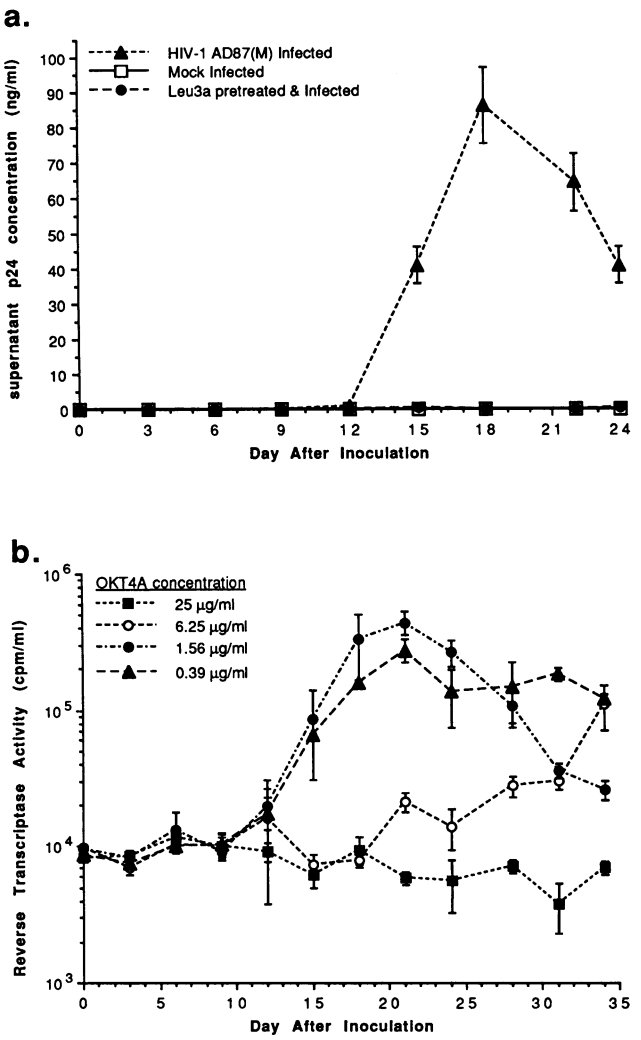


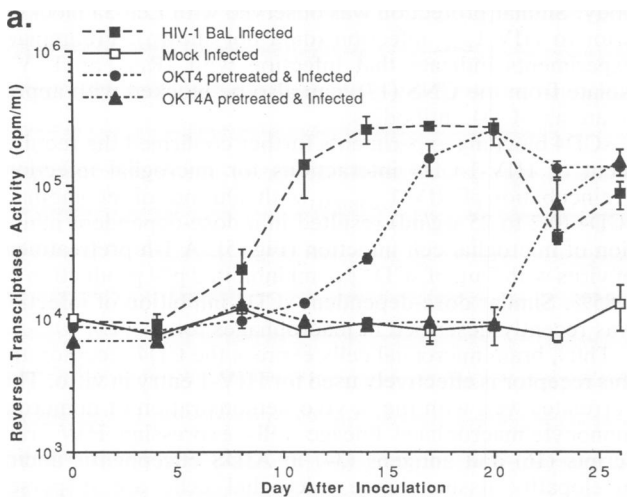
FIG. 3. HIV-$1_{AD87(M)}$ infection of microglial cells is blocked by anti-CD4 antibodies Leu-3a and OKT4A. Microglial cell cultures were prepared from normal cortex (a) or malignant glioma (b) as previously described (35). These cultures were infected with HIV-$1_{AD87(M)}$ at 14 days in vitro, using a standardized inoculum of 1×10^4 to 2×10^4 cpm of RT activity per 35-mm petri dish. Supernatant culture samples were harvested at the indicated times and assayed for RT and $p24^{gag}$ as previously described (35). Points represent the mean of two to four replicate samples, and error bars represent the standard deviation. (a) Cultures were pretreated with or without Leu-3a antibody (25 μg/ml) for 1 h at 4°C and then incubated with HIV-$1_{AD87(M)}$ for 1 h at 4°C in the presence of antibody. Cultures were then washed three times in Dulbecco modified Eagle medium (4°C) and refed with medium. (b) Cultures were treated with four different concentrations of OKT4A antibody (25, 6.25, 1.56, and 0.39 μg/ml) and infected as above.

RESULTS AND DISCUSSION

Human brain cultures were established either from temporal lobe, resected as a therapeutic measure in patients with intractable epilepsy, or from a resected malignant glioma (35). Microglial cells in these cultures were characterized by fluorescent staining for a number of monocyte/macrophage lineage markers including LDL-R and Fc receptor (Fc-R) (35). The temporal lobe biopsy cultures contained 40% microglia, while cultures enriched from glioma tissue contained up to 99% microglial cells (35). CD4 protein expression could not be demonstrated by immunofluorescence in these cells.

To determine whether the CD4 transcripts were expressed in microglial cells, we used a highly sensitive PCR-based technique. With this technique, we first analyzed temporal lobe biopsy tissue, which would be used to establish cultures, and postmortem brain tissues. A3.01 T-lymphocyte and primary monocyte/macrophage RNAs were included as positive controls because these cells express the CD4 recep-

tor (31). Rat brain RNA was included as a negative control since our CD4 primers correspond to regions of divergence between the human and rat CD4 genes. Transcripts for two monocyte/macrophage-specific genes, LDL-R and Fc-R, were detected in RNAs from the brain tissue and blood-derived macrophages but not in T cells as predicted (data not shown). CD4 transcripts were detected in RNA harvested from all human tissues (temporal lobe biopsy, five brain regions from autopsy material, and both T-cell and monocyte/macrophage preparations) but not in rat brain RNA (Fig. 1). Sequential PCR amplification with internally nested primers resulted in higher sensitivity of detection (27) and confirmed the presence of CD4-specific sequences. Restriction enzyme analysis of the PCR products yielded the expected size fragments (data not shown).



We then used these CD4-specific primers to analyze total RNA of human brain cultures containing microglial cells. PCR amplifications were also performed with β-actin primers to verify the efficiency of RNA extraction and cDNA synthesis. Expression of the microglial cell-specific LDL-R transcripts as well as CD4 transcripts (Fig. 2) was detected in both the mixed glial cultures and the microglia-enriched glioma cultures by a single round of PCR amplification (40 cycles). Nested-primer analysis confirmed the specificity of the initial amplification for both transcripts. In experiments in which very small amounts of RNA were extracted from the cells, the second round of amplification was required for CD4 transcript detection. Thus, the CD4 gene was expressed in human cortical cultures and in highly enriched microglial cultures derived from glioma tissue.

To determine whether HIV-1 utilizes the CD4 receptor to infect microglial cells as it does in T lymphocytes and monocyte/macrophages (2, 15, 28, 30), we performed block-

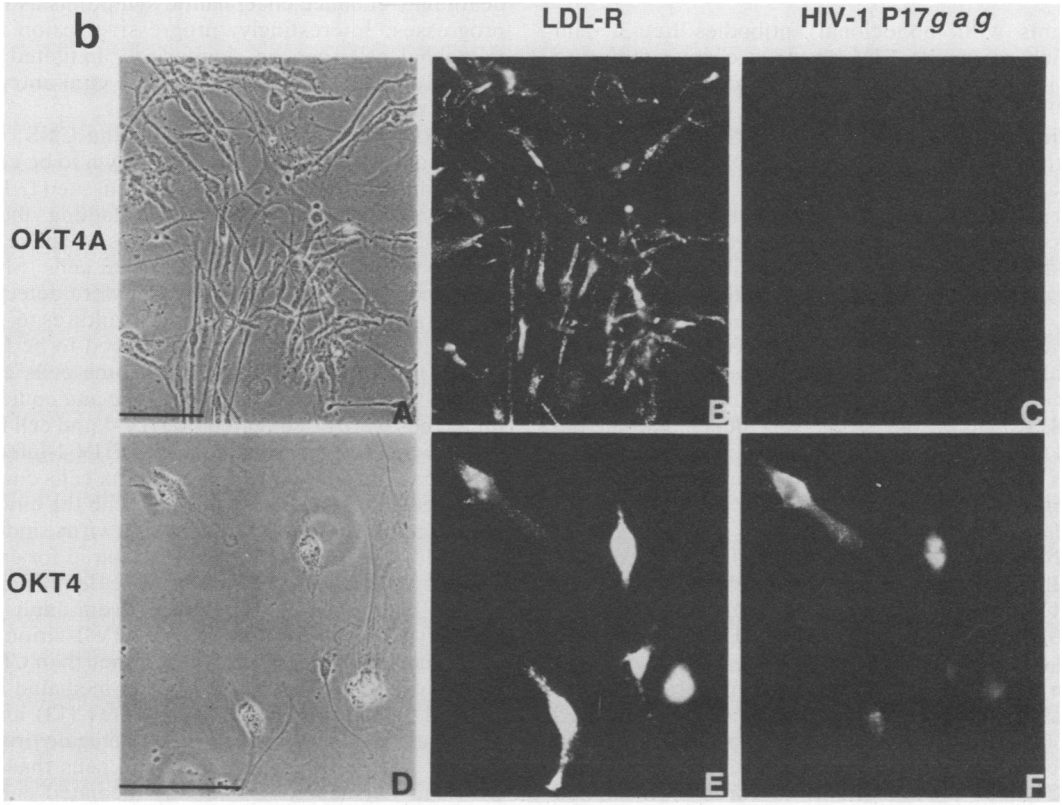


FIG. 4. HIV-1_{BaL} infection of microglial cells is specifically blocked by OKT4A. (a) Cultures were preincubated with 25 μg of OKT4A or OKT4 anti-CD4 antibodies per ml and then infected with 1×10^4 to 2×10^4 cpm of RT activity of HIV-1_{BaL} as described in the legend to Fig. 3. Control infected and mock-infected cultures were treated identically except the antibody or virus was omitted. (b) Some of the cultures represented in panel a (OKT4A pretreated, A to C; OKT4 pretreated, D to F) were fluorescently stained to reveal LDL-R (B and E) and HIV-1 $p17^{gag}$ (C and F) at 20 days after infection by previously described techniques (35). Panels A and D are phase-contrast views of the corresponding fields. Note that cultures pretreated with OKT4A do not show any signs of microglial cell death, while the OKT4-pretreated cultures contain reduced numbers of microglial cells which are enlarged and express HIV-1 $p17^{gag}$. Bars equal 100 μm.

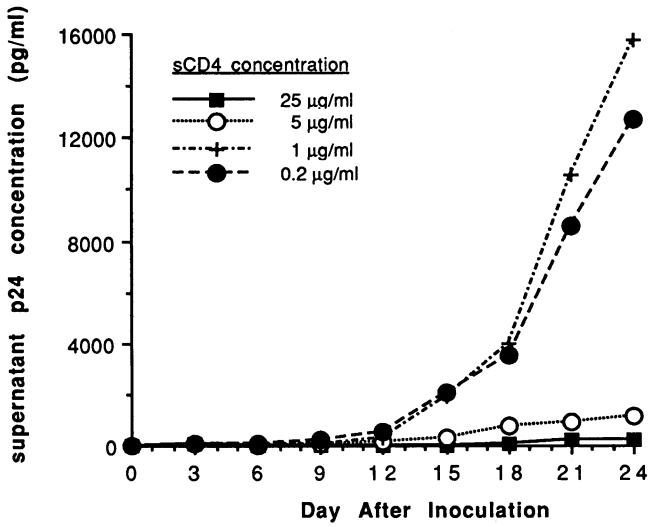


FIG. 5. HIV-1_{BaL} infection of microglial cells is blocked by sCD4. Human brain cultures were infected with HIV-1_{BaL} which had been pretreated with various concentrations of recombinant sCD4 (0.2, 1, 5, and 25 μg/ml) for 1 h at 4°C. Supernatant culture samples were harvested at the indicated times and assayed for $p24^{gag}$ as previously described (35). Points represent the mean of one to three replicate samples from a representative experiment.

ing experiments with monoclonal antibodies Leu-3a and OKT4A, which recognize CD4 epitopes corresponding to the binding site for the HIV-1 cell attachment glycoprotein gp120 (23, 30). The blocking was performed at 4°C to inhibit capping and internalization of the CD4-antibody complexes (23). Productive infection of microglial cell cultures by the macrophage-adapted strain HIV-$1_{AD87(M)}$ (28) was prevented by preincubation of the cultures with 25 μg of Leu-3a per ml, as assayed by RT or $p24^{gag}$ antigen release in the supernatant fluids (Fig. 3a). OKT4A antibody (25 μg/ml) pretreatment also blocked HIV-$1_{AD87(M)}$ infection of microglial cells (Fig. 3b). Preincubation with 6.25 μg of OKT4A per ml delayed the development of a productive infection by 15 days, while blocking was not seen with further dilutions of OKT4A antibodies (Fig. 3b). Thus, the efficiency of blocking productive HIV-$1_{AD87(M)}$ infection was dependent on the dose of CD4 antibody used during preincubation. Similar blocking was obtained with primary lymphocyte cultures pretreated with OKT4A and infected with HIV-$1_{AD87(M)}$ or HIV-1_{BaL} (data not shown). However, equal doses of OKT4A antibody more efficiently blocked the infection of microglial cells than that of T cells, possibly because lower numbers of CD4 molecules are present on the surface of microglial (26) or monocyte/macrophage (15, 28) cells compared with T cells.

Replication of HIV-1_{BaL}, another well-characterized macrophage-adapted isolate (11), in microglial cells preincubated with OKT4A (25 μg/ml) was delayed 12 to 15 days relative to that in infected cultures not pretreated with antibody (peak RT activity 26 days postinfection). Cultures preincubated with OKT4 antibody (25 μg/ml), which recognizes a CD4 epitope outside the gp120-binding domain, produced RT levels comparable to unblocked controls after only a slight delay (3 to 6 days; Fig. 4a). This delay of virus production in OKT4-treated cultures may be due to patching and internalization of CD4 at 37°C or to conformational changes induced by OKT4 binding at a distant site (30). The considerable delay in viral production seen in OKT4A-treated compared with OKT4-treated cultures is likely due to the specificity of OKT4A for the HIV-binding site on CD4. OKT4A- and OKT4-pretreated cultures were also examined by double fluorescence staining for LDL-R and $p17^{gag}$ (Fig. 4b). Viral $p17^{gag}$ was only seen in rare cells from cultures pretreated with OKT4A antibody, while 50% of the LDL-R^+ microglial cells express $p17^{gag}$ in cultures pretreated with OKT4 antibody. Similar protection was observed with Leu-3a blocking prior to HIV-1_{BaL} infection (data not shown). Preliminary experiments indicate that infection with JR_{FL}, an HIV-1 isolate from the CNS (17), can also be blocked with appropriate anti-CD4 antibodies.

sCD4 blocking experiments further confirmed the requirement of HIV-1-CD4 interactions for microglial infection. Preincubation of HIV-$1_{AD87(M)}$ with dilutions of recombinant sCD4 (0.2 to 25 μg/ml) resulted in a dose-dependent inhibition of microglial cell infection (Fig. 5). A 1-h pretreatment of virus with 5 μg of sCD4 per ml inhibited p24 production by >85%. Similar dose-dependent sCD4 inhibition of infection was recently described in macrophages (8).

Thus, brain microglial cells express the CD4 receptor and this receptor is effectively used for HIV-1 entry in vitro. This correlates well with the in vivo demonstration of numerous monocyte/macrophage lineage cells expressing HIV transcripts (16) and antigens (34) in AIDS encephalitis and/or myelopathy tissue. Since microglial cells are dispersed throughout the brain (26), their progressive fusion and cell death may enhance encephalitic symptoms as HIV infection progresses. Interestingly, progressive fusion and death of microglial cells can be significantly inhibited by anti-CD4 antibody treatment even after initial viral entry and replication in vitro (34a).

The other types of glial cells in the CNS, astrocytes or oligodendrocytes, have not been shown to be consistently or significantly infected in AIDS brain tissue (1, 16, 33, 34). In primary human brain cultures containing abundant astrocytes (35) and some oligodendrocytes, we have not observed the productive infection of these glial cells. Similarly, transient and/or low levels of infection were detected in glioma cell lines or fetal dorsal root ganglia cultures (6, 9, 17–19, 32), and in these cells virus entry appeared to be CD4 independent (4, 7, 12, 36). Even when glioma cells expressed the CD4 receptor from a transfected gene and could bind HIV-1, these cells were not infected by HIV-1 and cell fusion did not occur when they were mixed with HIV-1-infected lymphocytes (5). The CD4-transfected glioma cells could, however, be infected by HIV-1 pseudotyped with the envelope protein of an amphotropic murine leukemia virus, indicating that a receptor other than CD4 may be required for entry of HIV-1 in these glioma cells. It remains to be demonstrated whether normal human astrocytes derived from adult brain behave similarly toward HIV-1 infection. HIV-1 virions coated with antibodies can bind to a receptor other than CD4, the Fc-R. Two clear examples are the Fc-R-mediated entry of opsonized HIV-1 into blood monocytes (13) and into CD4-negative fibroblasts induced by cytomegalovirus infection to express the Fc-R (24). However, in cells that express both CD4 and Fc receptors, antibody-mediated enhancement of HIV-1 infection can be blocked with sCD4 or Leu-3a, which suggests that CD4-virus interactions are also required for entry of opsonized virus (37). Recent evidence indicates that CD4 may interact with the Fab portion of antibodies coating the virus (20).

At present, the importance of any receptor other than CD4 in the natural CNS infection by HIV-1 is unknown. The in

vitro evidence presented here suggests that CD4-mediated entry of HIV-1 into microglial cells is a significant mechanism of virus spread to the brain. Thus, therapeutic strategies aimed at disrupting CD4-gp120 interactions may be appropriate for clearing brain infection. Recent evidence indicates that a CD4 region involved in cell fusion lies outside the virus-binding site; a single point mutation in this region is sufficient to prevent HIV-1-induced syncytium formation and cell-to-cell transmission of the virus (3). CD4-based peptides corresponding to this region might inhibit HIV-1-mediated cell fusion and/or HIV-1 entry as has been shown with small peptide fragments designed to competitively inhibit HIV-1-CD4 interactions (CD4 residues 81 to 92) (25). Since these benzylated peptides are small lipophilic molecules potentially capable of entering cells (25), they might be able to cross the blood-brain barrier and have a therapeutic effect in the CNS.

In conclusion, we propose that CD4-mediated viral entry is a major factor in the spread of HIV-1 to the CNS and that therapies aimed at blocking the CD4 receptor may be appropriate to inhibit HIV-1 infection of the brain.

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