

Primary Isolated Human Brain Microvascular Endothelial Cells Express Diverse HIV/SIV-Associated Chemokine Coreceptors and DC-SIGN and L-SIGN

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Chemokines have received increasing attention due to their inhibitory activities on human immunodeficiency virus type-1 (HIV-1) and simian immunodeficiency virus (SIV) replication and the potential for chemokine receptors to assist in HIV-1/SIV entry into permissive cells. Besides CD4, which is the major receptor for HIV-1 and SIV, a number of chemokine receptors including but not limited to APJ, CCR3, CXCR4, and CCR5 may be coreceptors for HIV-1/SIV, not only in peripheral blood and lymphoid tissues but also in the central nervous system (CNS). The present studies reveal the lack of CD4, but the significant expression of various chemokine receptors, APJ, CCR3, CXCR4, and CCR5, plus C-type lectins DC-SIGN and L-SIGN on isolated primary human brain microvascular endothelial cells (MVECs). As these MVECs do not express CD4, this suggests a CD4-independent HIV/SIV entry/infection of these cells, which are the major cells constituting the human blood–brain barrier. We also found that chemokines for cognate chemokine receptors individually were unable to block binding of HIV-1 to brain MVECs. These results reveal that in primary isolated brain MVECs viral attachment is mediated by a possible previously unknown receptor(s) or by cooperative activity of various receptors. Moreover, mRNA transcripts for DC-SIGN/L-SIGN, as well as DC-SIGN protein expression, suggest the capability of MVECs to attach viral particles on cell surfaces, even though polyclonal antisera for DC-SIGN did not affect viral binding to these cells. These data will assist in further understanding lentiviral entry into the CNS. © 2002 Elsevier Science (USA)

Key Words: HIV-1; chemokine receptors; DC-SIGN; L-SIGN; endothelial cells; CNS; blood–brain barrier.

INTRODUCTION

Human immunodeficiency virus type-1 (HIV-1) infection leads to up-regulation of various chemokines in humans and simian brain tissues (Schmidtmayerova *et al.*, 1996; Westmoreland *et al.*, 1998). Elevated concentrations of β -chemokines in cerebrospinal fluid (CSF) of HIV-1-seropositive individuals also suggest a major role for chemokines and their cognate receptors in the pathogenesis of neuroAIDS (Cinque *et al.*, 1998b; Kelder *et al.*, 1998). Recent data have demonstrated that chemokine and chemokine-receptor expression may be extremely important in understanding a variety of diseases of the primate and human central nervous system (CNS). Chemokines, which were first shown as chemoattractant agents in the immune system and for hematopoietic cells, have now been demonstrated clearly to be expressed differentially in the CNS (Glabinski *et al.*, 1995). Moreover, a number of studies demonstrating that certain chemokines are capable of suppressing HIV-1/SIV infection, and that chemokine receptors function as co-receptors for HIV-1/SIV entry into permissive cells, have

assisted in revealing several previous mysteries of HIV-1 pathogenesis (BouHamdan *et al.*, 2001; Cocchi *et al.*, 1995; Deng *et al.*, 1996; He *et al.*, 1997; Virelizier, 1999). It has been reported previously that lentivirus infection of the CNS induces neurological diseases in both humans and animals (Kolson *et al.*, 1998). In particular, the presence of HIV-1 in CSF and CNS-based cells suggests the need for further exploration of viral entry into the brain and the role of the brain protective sheath, the blood–brain barrier (BBB), in the neuropathogenesis of HIV-1 infection.

The physiological functions of chemokines range from signal transduction to brain development and maintenance of normal brain homeostasis (Klein *et al.*, 1999; Lazarini *et al.*, 2000; Zheng *et al.*, 1999; Zhou *et al.*, 1998). As far as CNS pathology is concerned, chemokines are involved in a number of neurodegenerative disorders, such as multiple sclerosis, strokes, Alzheimer's disease, AIDS-related dementia, and brain tumors (Desbaillets *et al.*, 1994; Gironi *et al.*, 2000; Kuratu, 1999; Langford and Masliah, 2001; Lukiw and Bazan, 2000). Most of the chemokines' activities are mediated through their receptors, which are members of a superfamily of G-protein-coupled proteins (Baggiolini, 1998).

DC/L-SIGNs are type II membranous proteins with

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characteristic external mannose-binding, C-type lectin domains (Steinman, 2000). The isolation and characterization of these glycoproteins are based on their capacity to bind the glycan-rich HIV-1 envelope in a CD4-independent manner (Curtis *et al.*, 1992). The role of DC-SIGN and L-SIGN in HIV-1 pathogenesis has been highlighted by recent studies demonstrating the capability of X4, R5, and X4R5 HIV-1 strains to bind DC-SIGN and increase further viral transmission to permissive cells. Of note, viral binding and transmission were critically dependent on surface expression of these moieties in transmitting cells (Pohlmann *et al.*, 2001).

Therefore, to gain a better understanding of the roles of various receptors and ligands involved in HIV-1/SIV entry into the CNS, we analyzed the repertoire of their mRNAs, expressed by primary isolated human brain microvascular endothelial cells (MVECs). Furthermore, the mRNA expression findings were substantiated with immunocytochemical analyses of the respective mRNA product protein on the cell surface. The functionality of the independent receptor/ligands was explored with respective blocking agents for each of the chemokine receptors, as well as polyclonal anti-DC-SIGN antiserum.

Our studies reveal that primary isolated human brain MVECs express a number of diverse chemokine receptors, such as CCR3, CXCR4, CCR5, and APJ. Moreover, these cells also express DC-SIGN and L-SIGN glycoproteins, which are being actively investigated for their role in HIV-1 entry and infection. We were unable to block viral surface binding on primary MVECs with chemokine receptor cognate ligands, as well as with DC-SIGN polyclonal antiserum. We conclude that brain MVECs express diverse chemokine receptors known for potential HIV-1/SIV entry; however, none of these receptors independently can control the virion attachment on the surface of these BBB-based cells and cellular entry.

RESULTS

Reverse transcriptase-polymerase chain reaction (RT-PCR) analyses of HIV-1/SIV coreceptors on primary isolated human brain MVECs

The presence of HIV-1 in CSF and various CNS-based cells suggests that these cells are potential targets for HIV-1/SIV infection and as transfer moieties to other CNS elements (Cinque *et al.*, 1998a; Di Stefano *et al.*, 1997, 1998; Enting *et al.*, 2001; Hengge *et al.*, 1998; McArthur *et al.*, 1997; Price, 2000). It has become established that chemokine receptors, CXCR4 and CCR5, act as major coreceptors together with CD4 for HIV-1 entry into diverse target cells (Deng *et al.*, 1996). CD4-independent and chemokine (CXCR4, CCR5)-dependent infection of various cells suggests the capability of certain HIV-1 isolates to infect cells devoid of CD4 but with functional chemokine coreceptors (Edinger *et al.*, 1997b; Edwards *et al.*, 2001). The BBB, with its MVECs, is the first line of

defense against viral entry into brain. Of note, several previous reports suggest HIV-1 infection of human brain MVECs and astrocytes via CD4-independent mechanisms (Moses *et al.*, 1993, 1996, 1997; Moses and Nelson, 1994; Schweighardt *et al.*, 2001).

Based on these previous findings, we examined primary isolated human brain MVECs for the presence of chemokine receptor CCR3, CXCR4, CCR5, and APJ mRNAs. We also explored whether DC-SIGN and L-SIGN mRNAs exist in human brain MVECs. As internal controls, we utilized von Willebrand factor (Factor VIII), specific for endothelium, and GAPDH mRNA. The primers used for amplification of each receptor, as well as controls, are described in Table 1. Of note, our RT-PCR amplicon sizes were in agreement with expected sizes for each PCR product: von Willebrand factor, GAPDH, APJ, CCR3, CXCR4, CCR5, CD4, DC-SIGN, and L-SIGN at 323, 309, 481, 417, 522, 339, 462, 347, and 418 bases, respectively. To further confirm the specificity of each amplicon, we performed Southern blotting for each of the amplified products by utilizing an internal sense probe for the PCR product. Table 2 describes various probes used for Southern blotting analyses of each of the receptors' cDNA, as well as controls. As shown in Fig. 1, primary isolated human brain MVECs do not express CD4 mRNA, the major receptor for HIV-1 attachment and cell entry. However, primary MVECs express mRNA for APJ, CCR3, CXCR4, and CCR5 chemokine receptors (Fig. 1). These findings suggest that HIV-1 attachment and entry in human brain MVECs possibly involve a CD4-independent mechanism, utilizing either one or multiple chemokine receptors. These results corroborate the previous reports describing CD4-independent, CCR5-dependent infection of simian brain capillary endothelial cells (Edinger *et al.*, 1997a) and reports showing that human brain MVECs could be infected with HIV-1 (Mukhtar *et al.*, 2000; Mukhtar and Pomerantz, 2000).

For assessing differential expression of DC-SIGN and L-SIGN, we aligned cDNA sequences of DC-SIGN and L-SIGN. Figure 2 shows the alignment of these two cDNA sequences. As demonstrated in Fig. 2, it is possible to generate primers that can differentially amplify cDNA for DC-SIGN and L-SIGN. The respective sense and antisense primers used for differential amplification of DC-SIGN and L-SIGN are described in Table 1 and Fig. 2. As shown in Fig. 1, we observed transcripts for both DC-SIGN and L-SIGN in primary brain MVECs, even though the message for DC-SIGN was more predominant than L-SIGN. Thus, the expression of DC-SIGN and L-SIGN on MVECs could be advantageous for viral entry into the brain.

Immunocytochemistry for receptors on primary isolated human brain MVECs

To further confirm the presence of various HIV-1/SIV coreceptors on primary brain MVECs, we extended the

TABLE 1
Primers Used for Chemokine Receptor RT-PCRs from Human Brain MVECs

Gene	Accession No./Reference	Primer	Sequence	Amplicon size (bases)
von Willebrand's factor		Sense	5'GGCTCAAGGACCGGAAGCGA3'	323
		Antisense	5'GGCCTTGTCTCAGGGGCTG3'	
GAPDH	NM_002046	Sense	5'CGGAGTCAACGGATTGGTCGTAT3'	309
		Antisense	5'AGCCTTCTCCATGGTGGTGAAGAC3'	
APJ	O'Dowd <i>et al.</i> (1993)	Sense	5'TACACAGACTGAAATCCTCG3'	481
		Antisense	5'TGCACCTTAGTGGTGTCTCC3'	
CCR3	AF026535	Sense	5'ATAGCTGGAGGCATTTCCACAC3'	417
		Antisense	5'GAAGGAATGGGATGTATCTGCC3'	
CXCR4	AF025375	Sense	5'CCTCCTCTTTGTATCAGCCTT3'	522
		Antisense	5'CCAATGTAGTAAGGCAGCCAAC3'	
CCR5	U54994	Sense	5'CTTCATCATCCTCCTGACAATC3'	339
		Antisense	5'CTCTTCTTCTCATTTCGACACC3'	
CD4	M35160	Sense	5'GTGGCACCTGGACATGC3'	462
		Antisense	5'GGTCCCCACACCTACA3'	
DC-SIGN	ALIGN_000098.dat	Sense	5'GCTGACCCGGCTGAAGGC3'	347
		Antisense	5'GGTGAGCCGTCCACCCATT3'	
L-SIGN	ALIGN_000098.dat	Sense	5'CAGTGGCATCAGACTTTTTTC3'	418
		Antisense	5'CCTGGTAGATCTCCTGCA3'	

RT-PCR findings with immunocytochemical analyses of the cells for each of the receptors. Before immunocytochemical analyses were performed, each batch of primary isolated brain MVECs was characterized for expression of von Willebrand factor, a marker expressed on MVECs. Approximately >97% von Willebrand factor-positive cells were used for further exploration of chemokine receptors. Of note, in our experience, we have found that primary brain MVECs lose their endothelial markers with multiple passages (not illustrated). The present study's observations are based on MVECs only at the third passage. We utilized secondary antibodies alone as negative controls for these analyses.

A granular cytoplasmic expression of von Willebrand factor confirmed the purity of these cells (Fig. 3A). CCR5, CCR3, and CXCR4 were demonstrated on human brain MVECs in these studies (Fig. 3A). Among the three receptors demonstrated in Fig. 3A, we observed higher expression of CXCR4, as compared with CCR3 and CCR5. Although this observation cannot be further substantiated due to variations in antibody affinity with each

of the antigens, by utilizing different antibodies from various sources for each of the chemokine receptors, we obtained a constant and reproducible expression pattern (data not illustrated). Figure 3B shows immunocytochemical data that APJ, one of the chemokine receptors whose ligand, Apelin-36, has been described recently (Cayabyab *et al.*, 2000), and DC-SIGN are also produced and expressed on these cells. Of note, a specific antibody for L-SIGN is not yet available.

HIV-1 binding to brain MVECs treated with selected chemokines, soluble CD4, and polyclonal DC-SIGN antiserum

To explore the functional role of each chemokine receptor toward lentiviral binding on the surface of human brain MVECs, we blocked each receptor with inhibitory concentrations of each chemokine and then exposed them to X4, R5, and dual-tropic HIV-1 isolates. The inhibitory concentrations of chemokines were based on earlier reports (Cayabyab *et al.*, 2000; Cocchi *et al.*, 1995; He

TABLE 2
Probes Used for RT-PCR Amplicon's Southern Blotting from Human Brain MVECs

1. vWF	5'GCAAGATCGACCGCCCTGAAGCCTCCCGCA3'	(30-mer)
2. GAPDH	5'GGGCGATGCTGGCGCTGAGTACGTCGTGGA3'	(30-mer)
3. CD4	5'GGCGAGCTGTGGTGGCAGCCGAGAGGGCT3'	(30-mer)
4. APJ	5'CCTTCTTCTGCAAGCTCAGCAGCTACCTC3'	(29-mer)
5. CCR3	5'GACCATCTTCTGTCTCGTTCTCCCTCTGCTCG3'	(32-mer)
6. CXCR4	5'GGATCCCTGCCCTCCTGCTGACTATTCCCG3'	(30-mer)
7. CCR5	5'GGGGCTGGTCCCTGCCGCTGCTTGTATGGT'	(30-mer)
8. DC-SIGN	5'GCTGAGGAGCAGAAGTTCCTACAGCTGCAGT3'	(31-mer)
9. L-SIGN	5'CCAGAACCTGACCCAGCTTAAAGCTGCAGT3'	(30-mer)

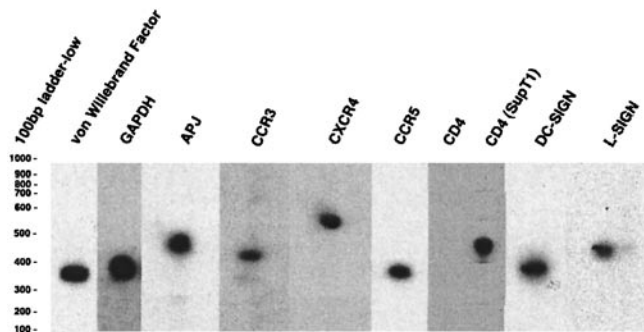


FIG. 1. RT-PCR analysis of various receptors' expression from primary isolated human brain MVECs. Various chemokine receptor transcripts were amplified from cDNA, generated from total RNA utilizing oligo(dT) primer. The specificity of each primer pair was confirmed by DNA PCR utilizing the respective plasmid DNA for each receptor. Lanes 1 and 2 show internal controls for von Willebrand factor and GAPDH mRNA. Lanes 3-6 include the chemokine receptors APJ, CCR3, CXCR4, and CCR5. Lane 5 is CD4 and lane 6 is an mRNA-positive control for CD4 from SupT1 T-cells. Lanes 7 and 8 show transcripts for DC-SIGN and L-SIGN. The size of each amplicon was further confirmed with the expected product size by Southern blotting. The hybridization probe for each amplicon spans the internal sequences of the amplicon. A 100-bp ladder-low from GenSura Laboratories (San Diego, CA) was used as a DNA size marker. This figure is representative of three independent studies.

et al., 1997). The APJ ligands, Apelin-36 (120 μ M), CCR3 ligand eotaxin (500 ng/ml), CXCR4 ligand SDF-1- α (2.5 μ g/ml), and a mixture of CCR5 ligands MIP-1- α MIP-1- β , and RANTES, each at a concentration of 500 ng/ml, were used for blocking respective chemokine receptors. For DC-SIGN, 1:5 diluted polyclonal anti-DC-SIGN antiserum was used, whereas a soluble CD4 concentration of 20 μ g/ml was utilized. The epitope used to generate the polyclonal antiserum is conserved between DC-SIGN and L-SIGN. Figure 4 reveals HIV-1 p24 antigen binding of various HIV-1 strains compared with an untreated control. As shown, none of the chemokines blocked viral binding on brain MVECs. Even though primary brain MVECs are devoid of CD4 receptor, we also used soluble CD4 to analyze the effects on virion binding on the surface of these cells. Interestingly, compared with controls we observed a larger amount of virus attached to soluble CD4-treated cells (see below).

We demonstrated mRNA, as well as protein, for DC-SIGN on primary isolated brain MVECs. Previously, DC-SIGN has been shown to attach viral particles to cells (Geijtenbeek *et al.*, 2000a,b; Pohlmann *et al.*, 2001). To investigate the role of DC-SIGN on brain MVECs, we utilized polyclonal DC-SIGN antiserum for blocking of this receptor before exposing these cells to viruses. It was shown that DC-SIGN antiserum did not block viral binding, further suggesting some yet unknown receptor involvement in binding of lentivirions to brain MVECs (Fig. 4).

We observed significantly enhanced binding of the macrophage-tropic (CCR5) Bal and dual-tropic (CCR5/

CXCR4) 89.6 virions on brain MVECs treated with Apelin-36, a ligand for the APJ receptor, and soluble CD4 (Fig. 4). Previously, Apelin-36 has been shown to inhibit viral replication of macrophage and dual-tropic HIV-1 strains (Cayabyab *et al.*, 2000). Nonetheless, there are no previous reports showing such a phenomenon in primary human isolated cells. However, in CD4⁺ T-cells, several CC-chemokines increased the replication of T-cell-tropic viral strains (Kinter *et al.*, 1998). This dichotomous effect of chemokines has been ascribed to a signaling mechanism through inhibitory guanine nucleotide-binding regulatory proteins. Whether a similar mechanism operates in brain MVECs requires further study.

The binding assay also reveals that only macrophage (R5) and dual-tropic (X4R5) virions could attach to human brain MVECs. As far as the X4-tropic strain NL4-3 was concerned, we did not observe any significant binding in the control, as well as in various ligand-treated cells (Fig. 4). These observations also support the previous reports

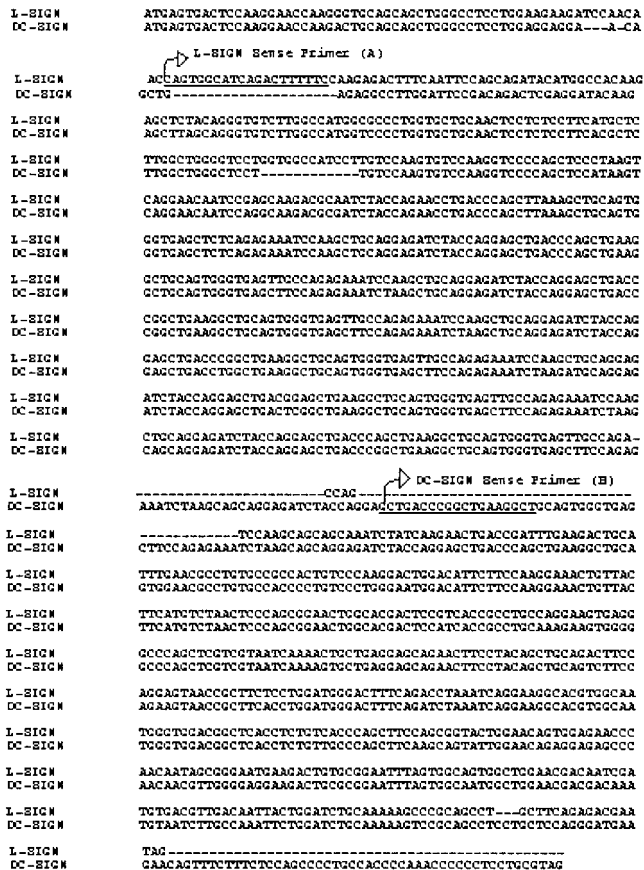


FIG. 2. Alignment of L-SIGN with DC-SIGN mRNA utilized to select primers for differential expression of L-SIGN and DC-SIGN from human brain MVECs. The forward arrow (A) shows the sense primer utilized to differentially amplify L-SIGN with an antisense primer from the homologous region of both mRNAs. Forward arrow (B) shows the DC-SIGN sense primer used to amplify only DC-SIGN mRNA transcripts. This alignment has been submitted to EMBL. The accession number for this alignment is ALIGN_000098.dat (alignment of DC-SIGN with L-SIGN cds. submitted by M. Mukhtar, April 5, 2001).

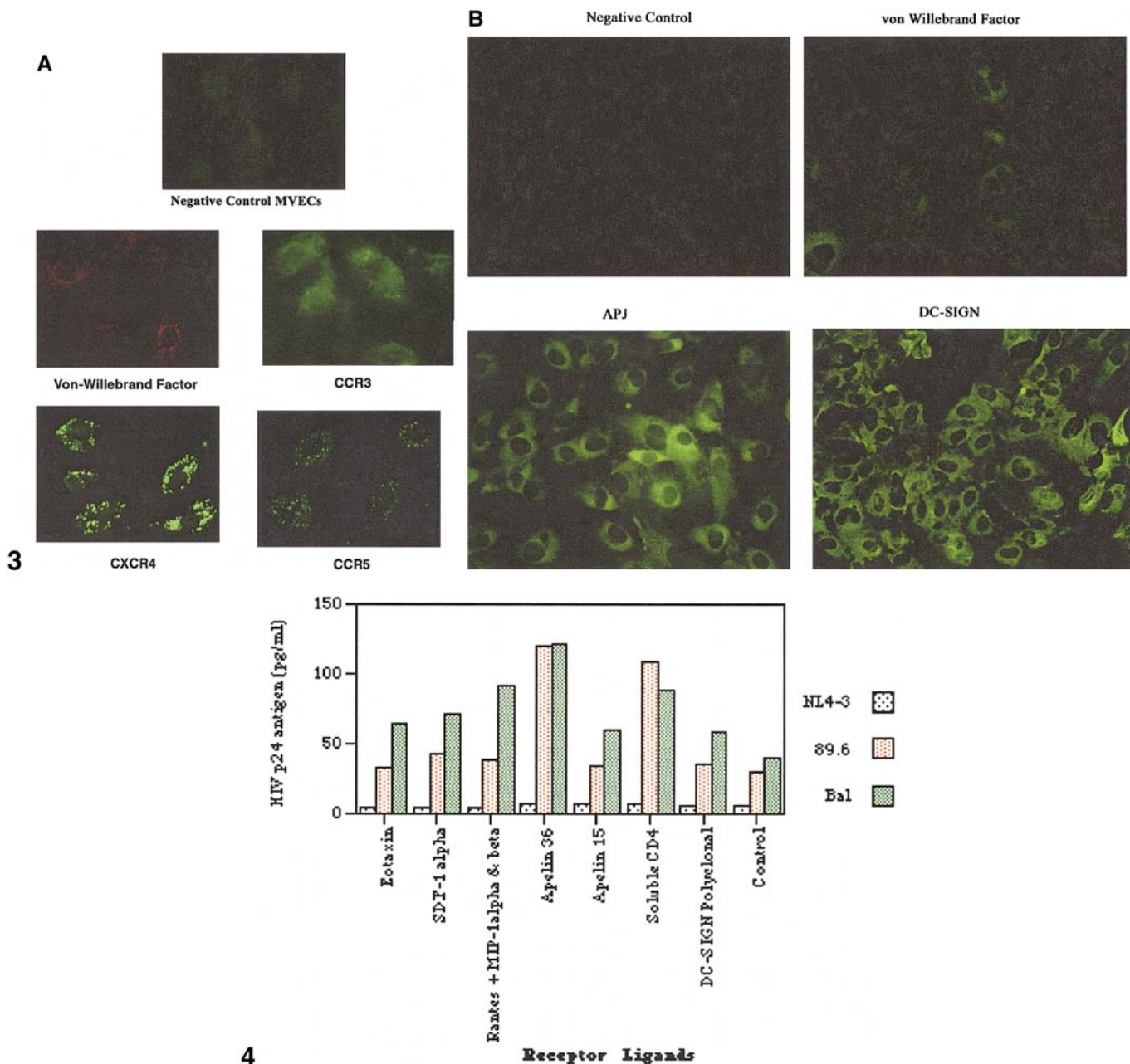


FIG. 3. (A) Immunofluorescence microscopy of primary isolated human brain MVECs for chemokine receptors. Primary isolated human brain MVECs were plated on two-well chamber slides and allowed to grow for 48 h. The cells were fixed with 3.5% formaldehyde and incubated with primary antibodies directed against respective chemokine receptors. Monoclonal antibodies were used for CCR3 (7B11), CXCR4 (12G5), CCR5 (2D7), and von Willebrand factor (Sigma). The secondary antibody used with the von Willebrand factor primary antibody is shown in red (Cy3); CCR3, CXCR4, and CCR5 were detected by Cy2 secondary antibodies and are shown in green. Both Cy2 and Cy3 were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). This figure is representative of three independent experiments. (B) Immunocytochemical analysis of brain MVECs for APJ and DC-SIGN. Anti-human APJ antibody was used to detect APJ in primary human brain endothelial cells. For detection of DC-SIGN, polyclonal antisera, kindly provided by Dr. Robert Dom's laboratory, University of Pennsylvania, were utilized. This figure is representative of two independent studies.

FIG. 4. Binding assays with cognate ligands for specific receptors. Effects of chemokines and polyclonal antisera on diverse HIV-1 strains' attachment to primary brain MVECs. Brain MVECs, actively growing in 6-well plates, were preincubated with the individual chemokines/polyclonal antisera, as shown, for 60 min. Plates were then incubated with normalized and equal quantities of HIV-1 p24 antigen (1.5 ng) equivalents for each indicated virus for 3 h. After 3 h, the cells were vigorously washed with $1 \times$ PBS and lysed with 0.5% Triton-X 100, and cell-associated HIV-1 p24 antigen was quantified via ELISA (Dupont). These findings are representative of two independent experiments.

suggesting mainly the presence of macrophage-tropic HIV-1 in human CSF and various CNS-based cells *in vivo* (Reddy *et al.*, 1996).

The above-described findings strongly suggest that chemokine receptors are clearly expressed in primary isolated human brain MVECs. None of these receptors

individually appears to be involved in HIV-1 attachment to the surface of these human BBB-associated cells.

DISCUSSION

The molecular mechanisms involved in HIV-1 binding to BBB-associated cells and entry into CNS remain enigmatic. CD4-independent, chemokine receptor-dependent infection and entry of SIV into simian capillary endothelial cells have been reported previously (Edinger *et al.*, 1997a,b, 1998; Reddy *et al.*, 1996). These data are further supported by our current results suggesting that CD4 is not detected on primary human brain MVECs, by RT-PCR as well as immunofluorescence microscopy assays. However, both CXCR4 and CCR5 are expressed on primary human brain MVECs, supporting their potential role in HIV-1 entry. Nevertheless, the present viral binding assays showed that none of the chemokine receptor ligands individually blocks HIV-1 attachment, suggesting that an alternative receptor or a cooperative action of known receptors is involved in viral attachment and entry.

The BBB consists mainly of microvascular endothelial cells and astrocytic foot processes and separates the CNS from the periphery (Joseph *et al.*, 1997; Mukhtar and Pomerantz, 1998; Rubin and Staddon, 1999). One of the major functions of the BBB is to ensure a constant internal environment for proper synaptic transmission and supply of essential nutrients for CNS-resident cells. This structure is continuously exposed to a variety of inflammatory cells, as well as infections in the body fluids.

HIV-1 frequently infects the CNS of individuals soon after seroconversion (Resnick *et al.*, 1988; Spector *et al.*, 1993). Initial studies have demonstrated that microglia and monocytes/macrophages are major cellular reservoirs for productive HIV-1 infection in the CNS (Bell *et al.*, 1993; Kure *et al.*, 1990; Pumarola-Sune *et al.*, 1987). A number of studies have also demonstrated limited replication of HIV-1, *in vivo* as well as *in vitro*, within MVECs, astrocytes, and neuronal elements utilizing immunohistochemistry and *in situ* polymerase chain reaction (Bagasra *et al.*, 1996; Mukhtar and Pomerantz, 2000; Nuovo and Alfieri, 1996; Nuovo *et al.*, 1994). It is a well accepted that highly infectious mononuclear cells, especially HIV-1-infected activated CD4⁺ T-cells, traverse the neural parenchyma and are probably much more efficient at delivering virus to perivascular macrophages in the brain. However, even a minor infection of brain MVECs may play a major role in compromising the BBB and easing the entry of free as well as cell-associated virus into the brain. Recent studies, showing differential cytokine and chemokine responses during neurological diseases induced by retroviruses, also suggest the potential role of chemokines and their cognate receptors in the neuropathogenesis of viral infections (Peterson *et al.*, 2001).

HIV-1 infection leads to a number of neurodegenerative disorders in AIDS patients. In the model of rhesus macaques infected with SIV, neurological disease manifestations correlate with replicative capacity of viral strains, as well as viral load in brain parenchyma (Demuth *et al.*, 2000; Rausch and Davis, 2001; Rausch *et al.*, 1994, 1999; Zink *et al.*, 1998, 1999), but the detailed neuropathogenesis of HIV-1 as well as SIV remains somewhat elusive.

Our present data and a number of other laboratory's findings suggest that primary isolated human brain MVECs are devoid of CD4 (Banks *et al.*, 1998). Moreover, it has also been suggested that CXCR4 on a variety of human endothelial cells can serve as the receptor for HIV-1 in the absence of CD4 (Molino *et al.*, 2000). Regarding CCR5, another predominant chemokine receptor facilitating the entry of R5 HIV-1 isolates, there are only a few reports suggesting CD4-independent, CCR5-dependent entry, though simian brain capillaries appear to be devoid of CD4 receptor and utilize a CCR5-mediated entry mechanism (Edinger *et al.*, 1997a,b, 1998). It has also been reported that CNS microglia express and utilize both CCR3 and CCR5 for infection of the CNS by HIV-1 (He *et al.*, 1997).

Higher expression of the chemokine receptor CXCR4 on primary isolated brain MVECs suggests a major role for this moiety in either signaling or viral entry. Most of the HIV-1 brain isolates show an R5 phenotype, suggesting CCR5-mediated entry/infection in CNS cells. Recently, it has been reported that HIV-1 isolates from brain and lymphoid tissues predict neurotropism independent of coreceptor specificity (Gorry *et al.*, 2001). Of note, CD4/CXCR4-independent infection of CNS-based cells by a T-tropic strain of HIV-1 has also been reported, suggesting alternative entry mechanisms irrespective of well-described chemokine receptors (Schweighardt *et al.*, 2001). These observations suggest certain previously unknown or cooperative actions of various chemokine receptors for entry/infection of CNS-based cells.

Recently, it has been shown that DC-SIGN as well as L-SIGN serve as an attachment factor for HIV-1 on cell membranes (Geijtenbeek *et al.*, 2000a,b; Pohlmann *et al.*, 2001). Moreover, DC-SIGN/L-SIGN-attached virus could exist stably bound or endocytosed. We identified cDNA regions in DC/L-SIGN which can be utilized to study differential expression of these genes (Fig. 2). Our studies also show that primary isolated human MVECs express both DC-SIGN and L-SIGN. As far as their role in viral attachment and entry into brain MVECs is concerned, we were unable to block the viral attachment on MVECs with polyclonal DC-SIGN antiserum. Currently, there is no potential DC/L-SIGN inhibitor available that could be utilized to completely discern the role of DC/L-SIGN expression on human brain MVECs; however, these ligands and their role in viral attachment/entry are vigorously being explored. The availability of potential

DC/L-SIGN inhibitors will also assist in understanding these ligands' expression on MVECs.

As stated above, chemokine receptors are seven-transmembrane domain, G-protein-coupled molecules that have drawn much attention in the recent past for their involvement in HIV-1 and SIV entry (Alkhatib *et al.*, 1996; Choe *et al.*, 2000; Feng *et al.*, 1996). Chemokines and their cognate receptors are also involved in the pathogenesis of immune-mediated inflammation of the CNS that controls leukocyte migration across brain endothelium, along with activation and movement of cells within the brain parenchyma (Baggiolini, 1998). In addition to these rapidly accumulating data on chemokines, there have been a number of reports evaluating chemokine-receptor expression in CNS-based cells. Various splicing patterns for chemokine-receptor isoforms (e.g., CXCR-4) have been found in mammalian astrocytes (Heesen *et al.*, 1997). Antigenetically, distinct conformations of CXCR4 further elaborate the hypothesis regarding the existence of multiple conformations of these coreceptors for viral entry (Baribaud *et al.*, 2001). CXCR-4 has also been demonstrated to be present on both microglia and astrocytes, initially in the mouse and rat (Heesen *et al.*, 1997; Tanabe *et al.*, 1997).

Among several other chemokine receptors, the role of APJ, also a seven-transmembrane chemokine receptor utilized for HIV-1 entry, is being explored. APJ shows higher efficiency for entry of X4 and R5X4 HIV-1 strains compared with R5 isolates (Edinger *et al.*, 1998). This coreceptor is highly expressed on the human neuronal precursor cell line (NT2)-generated postmitotic neurons, microglia, and oligodendrocytes (Choe *et al.*, 2000; Edinger *et al.*, 1998). A potential ligand for APJ, Apelin-36, with inhibitory activity for viral entry of primary T-cell-line-tropic (CXCR4) and dual-tropic (CXCR4 and CCR5) HIV-1 isolates has been recently described (Cayabyab *et al.*, 2000; Zou *et al.*, 2000). Of note, though, the inhibitory activity was studied only in cells expressing both CD4 and APJ.

Expression of various chemokine receptors and a number of other ligands on human brain MVECs is quite intriguing, as the BBB consists mainly of microvascular endothelial cells and is the first line of defense against viral entry into the brain. The presence of chemokine receptors and DC-SIGN/L-SIGN on BBB cellular constituents suggests the potential for these cells to attach to and endocytose HIV-1/SIV particles. Circulating HIV-1/SIV could utilize MVEC DC-SIGN/L-SIGN, protecting it from degradation and inactivation by immune regulatory cells. It has already been reported that DC-SIGN-bound HIV-1 has the advantage of increased stability and viability (Geijtenbeek *et al.*, 2000a,b). In a suitable environment, the MVEC-bound virus could enter the brain via capillary endothelial cells. Although these results are in conformity with our data and data from a number of other laboratories suggesting retroviral infection of endothelial

cells, we were still unable to block viral attachment on MVECs with ligands for APJ, CCR3, CXCR4, and CCR5. This suggests the possibility of some alternative receptors, such as proteoglycans (e.g., syndecans) or interaction of chemokine receptors with syndecan-like molecules (Saphire *et al.*, 2001; Valenzuela-Fernandez *et al.*, 2001). Further studies are necessary to detect the critical receptor(s) involved in penetration of the CNS by HIV-1/SIV *in vivo*.

In summary, the present results reveal that primary isolated human brain MVECs express a number of relevant chemokine receptors. The role of each chemokine receptor in viral entry has been elaborated previously. Expression of DC-SIGN and L-SIGN suggests the potential for primary brain MVECs to bind and protect HIV-1 on their membrane, as described earlier, in that DC-SIGN-bound virus is quite stable and infectious. It is hypothesized that under *in vivo* conditions circulating HIV-1 acts as an opportunistic pathogen, binds to brain microvasculature, and later, when the BBB is breached under the influence of various stimuli, enters the CNS parenchyma.

MATERIALS AND METHODS

Primary cells

Human brain MVECs were obtained from Cell System Corp. (Kirkland, WA). These cells are fed at 48-h intervals; however, when they reach 50% confluency they are fed every 24 h. It is essential to prewarm growth medium to 37°C for feeding the cells. These cells also required remarkably strict attention to proper conditions for *in vitro* culture. The passage reagents, trypsin/EDTA, EDTA, and the growth medium, must be warmed to 37°C. Ice-cooled trypsin inhibitor solution is used. (1) MVECs at >80% confluency are washed with EDTA solution. (2) The EDTA is replaced with a trypsin/EDTA mixture for 1 min. (3) Immediately the trypsin-inhibitor solution is added when the cells are dislodged from the flask surface; the supernatant is disposed and the pellets are suspended in prewarmed growth medium. (4) The cell suspensions are added to culture flasks precoated with attachment factor; precoating involves addition of attachment factor (AF) to the flask and wetting the whole surface with the AF. (5) The cells are incubated at 37°C, 5% CO₂, 100% humidity in human endothelial growth medium (Cell System Corp.). The purity of the cells is analyzed by immunofluorescence staining with antibody to von Willebrand factor, according to a previously described protocol (Mukhtar and Pomerantz, 2000).

Antibodies and chemokines

Anti-human von Willebrand factor and monoclonal anti-human CD4 were purchased from Sigma (St. Louis, MO). Monoclonal anti-APJ and anti-human CCR5 antibodies were purchased from R&D Systems (Minneapo-

lis, MN). An aliquot of CCR3 monoclonal antibody (7B11) was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (He *et al.*, 1997; Heath *et al.*, 1997). Anti-CXCR4 (12G5) antibody was a kind gift from Dr. James Hoxie's laboratory, University of Pennsylvania (Philadelphia, PA). Polyclonal DC-SIGN antiserum (1459) was kindly provided by Dr. Robert Doms, University of Pennsylvania (Soilleux *et al.*, 2002). The chemokines, recombinant human MIP-1 α , MIP-1 β , RANTES, eotaxin, and SDF-1 α , were purchased from R&D Systems. APJ ligands Apelin-36 and Apelin-15 were a kind gift from Dr. Shuju Hinuma of Takeda Chemical Industries, Ltd., Ibaraki, Japan.

PCR primers and probes

A complete listing of PCR primers, probes, and expected amplicon sizes is detailed in Tables 1 and 2. For selection of primers, we utilized Right Primer software, according to previously described specifications (Mukhtar and Bagasra, 1997). Of note, for DC-SIGN and L-SIGN primer selections, cDNAs for these genes were aligned to identify specific regions that could be utilized to differentially amplify both of these genes. An alignment for these genes has been submitted to the European Molecular Biology Laboratory (EMBL) under Accession No. ALIGN_000098.

Reverse transcriptase-polymerase chain reactions

Total cellular RNA from third-passage human brain MVECs was isolated by using the ULTRASPEC RNA Isolation System (Biotecx Laboratories, Inc., Houston, TX), according to the manufacturer's instructions. The integrity of total RNA was confirmed on an agarose gel. For cDNA synthesis, 4 μ g of total RNA was primed with oligo(dT) and reverse-transcribed in a 30- μ l reaction mixture utilizing the Sigma RT-PCR kit, according to the manufacturer's recommendations. Five microliters of cDNA was subjected to hot-start PCR amplification by using *Taq* polymerase. PCR conditions were an initial hot-start for 5 min at 94°C followed by 35 cycles of denaturing at 94°C for 45 s, annealing at 56°C for 45 s, and extension at 72°C for 1 min with a final extension at 72°C for 10 min. Both sense and antisense primers used for amplification of each receptor and amplicon size are shown in Table 1.

Viral stocks

The viral stocks used for infection of primary brain MVECs were produced by transfection of 293T cells with respective proviral DNA, as described previously (Ohagen *et al.*, 1999). Briefly, 48 h after calcium transfection of proviral DNA, supernatant was collected, filtered through a 0.45- μ m-pore-size filter, quantified by HIV-1 p24 antigen enzyme-linked immunosorbent assay (ELISA), and stored at -70°C for further utilization. The viral strains

used in these studies were NL4-3, an X4-tropic strain, Bal, an R5-tropic strain, and 89.6, an X4R5-tropic strain. Among these, NL4-3 utilizes CXCR4 as a major coreceptor, whereas R5 entry is mediated by CCR5, and dual-tropic strains use both CXCR5 and CCR5 for viral entry and infection.

Treatment of primary human brain MVECs with receptor ligands and HIV-1 p24 antigen-binding assays

Binding of viral particles to human brain MVECs was assessed by measuring cell-associated HIV-1 p24 antigen levels. Third-passage primary isolated human brain MVECs were seeded in 12-well plates and allowed to attach for 48 h. After 48 h, approximately 80% confluent brain MVECs were exposed to chemokine receptor ligands Eotaxin (500 ng/ml), SDF1- α (2.5 μ g/ml), RANTES, MIP-1- α , and MIP-1- β (500 ng/ml each), or Apelin-36 and Apelin-15 (120 μ M). For CD4 blocking, soluble CD4 (20 μ g/ml) was used, and 1:5 diluted polyclonal DC-SIGN antiserum was used for DC-SIGN blocking. After 60 min, each of the ligand solutions was decanted and the cells were exposed to 1.5 ng of p24 antigen equivalents of X4 (NL4-3), R5 (Bal), and X4R5 (89.6) viral strains in independent wells, with a control lacking all of the above ligands. After 3 h, the unbound virus was thoroughly washed off with 1 \times phosphate-buffered saline (PBS), and then the cells were lysed with Triton-X 100 and analyzed with HIV-1 p24 antigen ELISA (Dupont).

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