Neuropathogenesis of Lentiviral Infection in Macaques

Roles of CXCR4 and CCR5 Viruses and Interleukin-4 in Enhancing Monocyte Chemoattractant Protein-1 Production in Macrophages

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Neurological disease associated with lentiviral infection occurs mainly as a consequence of primary replication of the virus or a combination of the virus infection and replication of opportunistic pathogens in the central nervous system. Recent studies have shown that whereas the disease can be caused by CCR5 tropic viruses alone, its induction by CXCR4 (X4) tropic viruses occurred usually in association with infections caused by opportunistic pathogens and in the presence of a Th2 cytokine, interleukin (IL)-4.^{1,2} Further, X4-mediated neurological disease developed preferentially in rhesus compared to pig-tailed macaques. Because macrophages are the target cells for lentiviral infection in the brain and because macrophage chemoattractant protein (MCP)-1 is one of the major chemokines that is closely associated with acquired immune deficiency syndrome (AIDS) dementia, we tested for correlations between MCP-1 production and virus tropism in macrophages from the two species of macaques. The studies showed that the higher susceptibility of rhesus macaques to X4 virus-mediated encephalitis correlated with heightened production of virus and MCP-1 in cultured macrophages from this species and that these effects were

further enhanced with treatment with IL-4. However, the latter effect was restricted to macrophages infected with X4 viruses. IL-4 may therefore be a basic requirement for X4 viruses to cause central nervous system disease. (*Am J Pathol* 2002, 161:813–822)

One of the major biological properties of all members of the lentivirus taxonomic group is that irrespective of virus-host pair interactions, the ability of the virus to replicate productively in macrophages of the host is fundamental to the susceptibility of the individual to neurological disease.^{3–7} In human beings infected with human immunodeficiency virus (HIV), the human lentivirus, neurological disease has been associated primarily with infection of brain macrophages with viruses that use the CCR5 (R5) rather than those that use the CXCR4 (X4) co-receptor for entry into cells.^{8,9} Studies on replication of the two types of viruses in cultured macrophages have shown that the R5 viruses replicate much more efficiently in these cells than the X4 agents, and thus provided a rationale for the presence of R5 viruses in affected brains. This basic observation has been confirmed and extended in the macaque model of the neurological syndrome using SIV_{mac},^{6,7} and SIV_{smm}PGM¹⁰ (R5 viruses). However, neuropathology has also been observed in rhesus (Rh) macaques infected with the X4 tropic SHIVKU^{11,12} and the X4/R5 dual-tropic SHIV_{89.6}P.^{1,13} The two R5 viruses, SIV_{mac}251 and SIV_{smm}PGM, are highly macrophage-tropic in cultured macaque macrophages^{10,14} and are also associated with primary lentiviral encephalopathy^{6,10,11,15} unlike the X4 simian human immunodeficiency viruses (SHIVs) that cause encephalopathy, usually in association with other opportunistic pathogens.²

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Further studies aimed at exploring whether X4 virus-mediated neurological disease required synergy between the lentivirus and the opportunistic pathogen demonstrated that the infection of macaques with SHIVs, supplemented with injection of *Schistosoma mansoni* eggs (an experimental surrogate of opportunistic pathogens), into the animals resulted in development of lentiviral lesions in various tissues, including the central nervous system (CNS). These lesions were associated with heightened virus replication in macrophages in *S. mansoni*-induced granulomas that were rich in cells secreting Th2 cytokines, interleukin (IL)-4 and IL-10.¹ This suggested that the neuropathogenesis of X4 virus infections may be contingent on the presence of Th2 cytokines in the brain.

Histologically, characteristic pathological findings of lentiviral encephalitis consist of microglial foci, dense infiltration of mononuclear cells (including monocyte/macrophages) in the perivascular sites and in the brain parenchyma, and formation of syncytia comprised mainly of infected, fused cells bearing the macrophage marker.^{16–19} Although the mechanisms leading to monocytic infiltration in the CNS still remain unclear, it is speculated that secretory products, including CC chemokines, released by HIV-1-infected cells could be potentially important for recruitment of monocytes from blood to the brain. One such CC chemokine, monocyte chemoattractant protein-1 (MCP-1), has been shown to be the most potent of a variety of monocyte chemoattractants, including RANTES, macrophage inflammatory protein-1 α (MIP-1 α), MIP-1 β , MCP-2, and MCP-3, at inducing the transmigration of monocytes across a model of the blood-brain barrier.²⁰ The role of MCP-1 in monocyte recruitment *in vivo* in many organs, including the brain, has been supported by studies in transgenic mice overexpressing MCP-1^{21–23} and in CCR2-deficient mice.^{24–26} The selective accumulation of MCP-1, but not other chemokines, in the brains of patients with AIDS dementia,²⁷ in the cerebrospinal fluid (CSF) of AIDS patients with cytomegalovirus (CMV) infection,²⁸ and HIV-encephalitis²⁹ and in CSF of macaques with SIV encephalitis³⁰ further supports the role of this chemokine in the pathogenesis of lentiviral neurological disease. In addition, the role of soluble factors such as HIV Tat that modulate the expression of MCP-1, and other β chemokines in microglia and astrocytes, lends further support to the complex interplay of viral infection, chemokines, and chemokine receptors in the inflammatory processes of HIV encephalitis.³¹

In the present study, we examined whether there was a connection between the tropism of the virus for macrophages and the ability of the cells to produce MCP-1. The experiments confirmed that the higher susceptibility of Rh compared to pig-tailed (Pt) macaques for development of encephalitis correlated with the ability of macrophages from the Rh species to produce more virus and higher concentrations of MCP-1 than Pt macaques. The Th2 cytokine, IL-4 caused further enhancement of production of MCP-1 in macrophages from both species, but its enhancing effect on virus replication was confined to Rh macrophages infected with X4 viruses.

Materials and Methods

Viruses

We obtained SHIV-4 DNA encoding the *env*, *tat*, *rev*, and *vpu* genes of HIV-1 HXBc2 on a background of SIV_{mac239}³² from Dr. Joseph Sodroski, Harvard University, Boston, MA. Viral DNA was transfected into CEMx174 cells to produce a virus that was used to initiate sequential passages in macaques. Virus isolated from cerebrospinal fluid obtained 8 weeks after inoculation from Pt macaque PNB in passage four was amplified in a culture of peripheral blood mononuclear cells from a normal macaque and subsequently designated as SHIV_{KU-1}.³³ A further passage of this virus in Rh macaques gave rise to SHIV_{KU-2}.³⁴ SHIV_{89.6P} was kindly provided by Dr. N. Letvin (Harvard University), SIV_{mac}251 was a gift from Dr. R. Desrosiers (Harvard Medical School) and SIV_{smm}PGM from Dr. F. Novembre (Emory University, Atlanta, GA).

Macrophage Cultures

Peripheral blood mononuclear cells from macaques were obtained by Ficoll-Hypaque (Sigma, St. Louis, MO) gradient centrifugation and suspended at a concentration of 2×10^6 cells/ml in macrophage differentiation medium consisting of RPMI medium supplemented with 20% heat-inactivated human serum, 5 U/ml of M-CSF (Genetics Institute), 100 U/ml of GM-CSF (Genetics Institute, Cambridge, MA) and 5% heat-inactivated Rh monkey serum. Six-well dishes (Costar, Cambridge, MA) were seeded with 3 ml of medium containing 6 to 10×10^6 cells/per well and incubated overnight at 37°C. Cultures were then rinsed to remove nonadherent cells, refed with macrophage differentiation medium, and maintained for 7 days to allow adherent monocytes to differentiate into mature macrophages. Cells in our cultures were exclusively macrophages because >98% of them expressed the CD14 monocyte/macrophage-specific cell surface marker (data not shown). These cells were then inoculated with cell-free SHIV_{KU-2}, SHIV_{89.6P}, or SIV_{mac}251 at an multiplicity of infection (MOI) of 0.1 for 24 hours at 37°C. Cultures were then rinsed three times with R10 and replenished with macrophage medium. Studies were performed on macrophage cultures from at least three animals of either species and each experiment was performed in triplicate.

Cytokine Treatment and Virus Assay

Inoculated cultures were maintained in the presence or absence of recombinant macaque (rm) IL-4 at a concentration of 1.5 U/ml.³⁵ The medium was replenished every third day with fresh rm IL-4. Supernatant fluids were collected at regular intervals for determination of MCP-1 content by enzyme-linked immunosorbent assay (ELISA) and virus concentrations using reverse transcriptase (RT) assays. Viral replication was monitored by assaying the RT activity in the supernatants as described previously.³⁶ Macrophages from normal animals were infected with the

Table 1. Primers for Semiquantitative RT-PCR Analyses of Chemokine Receptor mRNA

Receptor	Primer	Position	Size of PCR product (bp)
CCR5	Forward: 5'-ATG ACT GAC ATC TAC CTG CTC-3'	194–214	416
	Reverse: 5'-CAT TAA AGA TGG TCA TCT TGG GG-3'	588–610	
CXCR4	Forward: 5'-CTC ATT TCA ATA GGA TCT TCC TGC C-3'	101–125	483
	Reverse: 5'-CTT CTA CCC CAA TGA CTT GTG-3'	564–584	
β -Actin	Forward: 5'-AAC ACC CCA GCC ATG TAT GT-3'	105–124	514
	Reverse: 5'-AGC ACT GTG TTG GCG TAC AG-3'	600–619	

CCR5 gene bank access number: [gi 2305193 gb AF011538.1](#); CXCR4 gene bank access number: [gi 1771982 gb U73740.1 MMU73740](#); β -actin gene bank access number: [gi 6636343 gb AF209434.1 AF209434](#).

X4 and R5 viruses as described above followed by treatment with or without rm IL-4 for 7 days. The medium was replenished every third day with fresh rmlL-4 (1.5 U/ml). Studies were performed on macrophage cultures from at least three animals of either species and each experiment was performed in triplicate.

Detection of MCP-1

MCP-1 was detected by ELISA (R & D systems, Minneapolis, MN). The ELISA could detect concentrations as low as 10 pg/ml. Protein measurements were determined by comparison to a standard curve, run in duplicate with each assay.

Semiquantitative RT-Polymerase Chain Reaction (PCR) Analyses of CXCR4 and CR5 mRNAs

For semiquantitative RT-PCR, RNA from Rh and Pt macaques, with and without IL-4 treatment, was extracted with Trizol reagent (Life Technologies, Inc., Grand Island, NY), and co-receptor mRNA levels were expressed as a function of the amount of β -actin mRNA for each RNA sample.³⁶ The sequences of primers used for RT-PCR reactions are shown in Table 1. For RT reactions, 1 μ g of total RNA from each sample was used in the Titan One-Tube RT-PCR system (Boehringer-Mannheim, Indianapolis, IN). Presence of any DNA contamination in the RNA preparations was tested by performing duplicate reactions, and subjecting one of them to 99°C for 2 minutes followed by 3 minutes at 95°C, to inactivate the RT activity. When present, DNA was removed with DNase I (Life Technologies, Inc., Gaithersburg, MD), followed by extraction. The reactions were performed in a Perkin-Elmer (Emeryville, CA) DNA Thermal Cycler 480 with a temperature profile of 42°C for 30 minutes, one cycle; 94°C for 5 minutes, one cycle; 94°C for 30 seconds, 55°C for 30 seconds, 68°C for 45 seconds, 10 cycles; 94°C for 30 seconds, 55°C for 30 seconds, 68°C for 45 seconds, 5-second extension/cycle, 30 cycles; 68°C for 6 minutes. PCR products were resolved by electrophoresis in 1%

agarose gels (SeaKem MR; FMC BioProducts) in 0.04 mol/L of Tris-acetate (pH 8.5) and 0.001 mol/L ethylenediaminetetraacetic acid containing 0.05 μ g of ethidium bromide per ml. The UV fluorescence of cDNA bands was measured with a Chemilmagerv5.5 Digital Imaging System. Data are presented as measured fluorescence of co-receptor cDNA/measured fluorescence of β -actin cDNA. Studies were performed on macrophage cultures from at least three animals of either species and each experiment was performed in triplicate.

Statistical Analysis

Statistical analysis was performed using one-way analysis of variance.

RNA Studies on Archival Brain Tissues

Archival brain tissue from uninfected Rh macaques and macaques that developed encephalitis after infection with SHIV_{89.6}P and treatment with *S. mansoni* eggs were used in this study. RNA was isolated by homogenizing weighed portions of brain tissues in Trizol (Life Technologies) followed by precipitation with isopropanol. Air-dried RNA pellets were then resuspended in distilled water and their concentrations determined spectrophotometrically. RNA integrity was confirmed by fractionation on 1.2% (w:v) agarose-formaldehyde gels and staining the ribosomal bands with ethidium bromide. For slot blot analysis, 0.6- to 5.0- μ g aliquots of RNA were applied in a final 200 μ l solution of 6.15 mol/L formaldehyde and 10 $\times$ standard saline citrate (1.5 mol/L sodium chloride and 0.15 mol/L sodium citrate, pH 7.0) onto a Hybond-N+ nylon membrane (Amersham, Arlington Heights, IL). An equal amount of ribosomal RNA was loaded onto the slot blot and used as a negative control. The specificity of the slot blot was confirmed by performing Northern analysis. Total RNA (20 μ g) from deep white matter regions of the brains from normal and SHIV_{89.6}P-infected Rh macaques with encephalitis was denatured in a loading buffer containing 2.2 mol/L of formaldehyde for 5 minutes at 65°C and electrophoresed in 1% (w:v) agarose gels containing

0.66 mol/L of formaldehyde. The RNAs were transferred to nylon membrane using the capillary transfer technique. After transfer the blots were air-dried and fixed in a UV crosslinker (Stratagene, La Jolla, CA). The probe was labeled with deoxycytidine 5'-[α - 32 P]triphosphate by a random-primed labeling system (Amersham), with a specific activity of 3.0×10^9 counts/min/ μ g DNA. Prehybridization (2 hours) and hybridization (overnight) conditions were performed as described.³⁷

Studies on the Brain

Animals were deeply anesthetized and CSF was withdrawn from the cisterna magnum. The animal was then exsanguinated by aortic cannulation and the vasculature perfused with 1 L of normal saline. The brain and spinal cord were removed and the brain bisected sagittally. The right half was transferred into 10% buffered formalin and the left half was dissected into nine anatomically distinct regions. All samples were snap-frozen in methylbutane chilled in liquid nitrogen and stored at -70°C . Portions of the left half of the brain and the entire right half were kept in buffered formalin for 3 to 4 days. Paraffin sections were stained with hematoxylin and eosin as a preliminary screen for morphological abnormalities, and serial sections were stained with Luxol Fast Blue and Sevier Munger stains for assessment of suspected myelin and axonal abnormalities, respectively.¹¹ The criterion for choosing SHIV_{89.6}P-infected macaque brains for this study stems from our earlier findings¹ that a predictable CNS pathology developed in infected animals that had been inoculated subcutaneously with eggs of *S. mansoni*.

Immunohistochemistry

Paraffin sections were used for immunohistochemical analysis to identify cell-specific markers. To detect the macrophage-specific marker, CD68, sections were treated with CD68 (DAKO, Carpinteria, CA) followed by treatment with biotinylated goat anti-mouse IgG (DAKO), peroxidase-conjugated streptavidin (DAKO), and NovaRed substrate (Vector Laboratories, Burlingame, CA), which yields a reddish reaction product.^{11,38}

In Situ Hybridization Studies

In situ hybridization was performed on paraffin-embedded brain tissue sections (deep white matter) from two Rh macaques with lentiviral encephalitis and two normal controls (archival tissues described above). *In situ* hybridization was performed as previously described.³⁹ Sections were air-dried for 10 minutes, then fixed in 4% paraformaldehyde at 4°C for 15 minutes. Slides were rinsed twice in phosphate-buffered saline for 5 minutes, acetylated, and hybridized at 45°C for 4 hours in 50% formamide buffer containing the ^{35}S -labeled sense or anti-sense cRNA of macaque *gag*⁴⁰ and MCP-1 probe. Macaque-specific MCP-1 sequences were amplified by PCR from a human MCP-1 cDNA (GenBank no. M37719) and subsequently cloned in a pGEMT7z vector for use as

Figure 1. Reverse transcriptase activity in supernatant fluids of macrophage cultures from Rh (A) and Pt (B) macaques inoculated with SHIVkU-2 (■), SHIV89.6P (●), and SIVsmmPGM (▲). Macrophage cultures from three animals of each species were inoculated with each of the three viruses and analyzed in triplicate. Error bars represent standard deviations. *, Highly significant levels ($P < 0.05$, one-way analysis) compared to day 0 cultures.

riboprobe in these studies. After hybridization and washing, slides were incubated with ribonuclease A (20 μ g/ml) at 37°C for 15 minutes, and ribonuclease-resistant hybrids were detected by autoradiography, using Kodak NTB-2 liquid emulsion (Eastman Kodak, Rochester, NY). Silver grains indicated the sites of accumulation of the MCP-1 mRNA. Slides were poststained with hematoxylin.

Results

Comparative Replicative Efficiencies of X4 and R5 Viruses in Macrophages Derived from Rh and Pt Macaques

Because the R5 viruses are known to cause encephalitis in both Rh and Pt macaques, we compared the replication efficiencies of two members of each viral type in cultures of macrophages derived from the two species of macaques. Monocyte-derived macrophages (MDMs) from both macaque species were inoculated with X4 (SHIV_{KU-2}), X4/R5 (SHIV_{89.6}P), and R5 (SIV_{smm}PGM) viruses at a multiplicity of 0.1 and supernatant fluids examined for virus content at various times after inoculation. As seen in Figure 1, A and B, whereas the R5 virus replicated efficiently in cells from both species of animals, the

Figure 2. MCP-1 ELISA analysis of supernatant fluids from uninfected macrophages and from SHIVku-2, SHIV89.6P-, SIVmac251-, and SIVsmmPGM-inoculated MDMs from Rh (A) and Pt (B) macaques. Samples were collected on days 4, 11, and 14 after inoculation. Data are shown as mean $\pm$ SE for three replicates. *, Highly significant levels ($P < 0.05$, one-way analysis of variance) compared to day 0 cultures.

X4 viruses replicated more efficiently in Rh cells than in Pt cultures. These studies were performed using MDMs derived from blood of at least three animals of each species. Each experiment was performed in triplicate.

SHIV_{KU-2}, SHIV_{89.6P}, SIV_{mac}251, and SIV_{smm}PGM Infections Superinduce MDMs to Secrete MCP-1

To investigate whether infection in macrophages with the viruses would result in stimulation of the cells to produce MCP-1 and whether the amount produced varied between the viruses and the species of host cells, we inoculated Rh and Pt MDMs with X4 (SHIV_{KU-2}), X4/R5 (SHIV_{89.6P}), and two R5 (SIV_{mac}251 and SIV_{smm}PGM) viruses. Supernatant fluids from the cultures were collected sequentially and examined for content of MCP-1 by ELISA on different days after inoculation. MCP-1 was constitutively secreted by uninfected MDM from both species at approximately the same levels. However, there was a clear-cut up-regulation of secretion of the chemokine in cultures infected with all four viruses (Figure 2, A and B). Among these, infected Rh MDM secreted higher levels of MCP-1 than corresponding Pt cells. In addition, the R5 viruses stimulated more MCP-1 than the

X4 viruses. These observations correlated well with the higher susceptibility of Rh and Pt macaques to neurological disease after infection with the R5 viruses, and the low susceptibility of Pt macaques to the syndrome after infection with X4 viruses.^{2,11}

MCP-1 Secretion from MDM Requires Virus Replication

To confirm that MCP-1 production requires replication of the virus rather than mere binding of the agent to cellular receptors, we inoculated Rh and Pt MDM cultures with live and heat-inactivated (56°C for 30 minutes) SHIV_{KU-2}, SHIV_{89.6P}, SIV_{mac}251, and SIV_{smm}PGM viruses, respectively, and examined supernatant fluids at day 14 after inoculation for the presence of MCP-1. The data showed that the concentration of MCP-1 secreted from Rh MDM cultures in the presence of heat-inactivated virus was identical to the low constitutive level of MCP-1 secreted by uninfected MDMs but that infection in the cells with the viruses resulted in massive production of the chemokine (data not shown). A similar result was observed for Pt MDMs. These findings proved that enhanced production of MCP-1 required productive viral infection in the cells.

Exogenous IL-4 Enhances Replication of X4 But Not R5 Viruses

To reconcile the data that the X4 viruses can cause encephalitis in Rh macaques,² we investigated the virus-host-macrophage interactions under conditions that simulated the pathological findings in the macaques. Because enhanced replication of SHIV_{89.6P} in tissue macrophages has been shown to be associated with increased Th2 cytokines,¹ we asked whether IL-4 could cause enhancement of virus replication in cultured macrophages. MDMs from both species of macaques were inoculated with the X4 (SHIV_{KU-2}), X4/R5 (SHIV_{89.6P}), and the R5 (SIV_{mac}251) viruses in the presence of exogenous recombinant macaque IL-4. As shown, IL-4 caused enhancement of replication of only the X4 viruses (compare to values reported in Figure 1), almost exclusively in Rh MDMs (Figure 3A) because differences were not significant in Pt MDMs (Figure 3B). The cytokine caused no enhancement of replication of the R5 viruses in macrophages from either species. These studies were performed using MDMs derived from blood of at least three animals of each species. Each experiment was performed in triplicate.

Exogenous IL-4 Superinduces MCP-1 Production in Uninfected and X4 Virus-Infected MDMs

We next asked about the effects of IL-4 on MCP-1 production in uninfected macrophages from the Rh (Figure 4A) and Pt (Figure 4B) macaques and in parallel cultures that had been inoculated with X4, X4/R5, and R5 viruses and cultured in the presence of IL-4. The results showed

Figure 3. Reverse transcriptase activity in supernatant fluids of macrophage cultures from Rh (A) and Pt (B) macaques inoculated with SHIVKU-2 (■), SHIV89:6P (●), and SIVsmmPGM (▲) in the presence of recombinant macaque IL-4 (1.5 U/ml). Macrophage cultures from three animals of each species were infected with each of the three viruses and analyzed in triplicate determinations. Error bars represent standard deviations. *, Highly significant levels ($P < 0.05$, one-way analysis of variance) compared to day 0 cultures.

that uninfected MDM treated with IL-4 consistently secreted substantially greater concentrations of MCP-1 (3.2- to 12.4-fold) than untreated cultures from either species. The cytokine however, had much more dramatic effects in Rh MDMs infected with the X4 viruses. Here, IL-4 caused more than a twofold higher production of MCP-1 than in parallel cultures that were not treated with the cytokine (compare with Figure 2). The cytokine also had an additive effect on MCP-1 production in X4-infected Pt MDMs, but this was minimal. Interestingly, comparison of R5 virus replication in MDMs in the absence (Figure 2) or presence of IL-4 (Figure 4) showed that the cytokine had minimal additive effects on MCP-1 production beyond levels caused by infection alone.

Exogenous IL-4 Enhances CXCR4 But Not CCR5 mRNA Expression

To understand the mechanism of IL-4-induced selective up-regulation of X4 but not R5 virus replication, we evaluated the expression of CXCR4 and CCR5 mRNAs in MDMs from Rh and Pt macaques cultured in the presence or absence of exogenous IL-4 using semiquantitative RT-PCR. Figure 5 shows that uninfected Rh MDMs expressed a higher level of CXCR4 mRNA compared to Pt MDMs, and that the expression in Rh MDMs was

Figure 4. MCP-1 ELISA analysis of supernatants from uninfected macrophages and from SHIVKU-2-, SHIV89:6P-, SIVmac251-, and SIVsmmPGM-inoculated MDMs cultured in the presence of rm IL-4 (1.5 U/ml) from Rh (A) and Pt (B) macaques on days 4, 11, and 14 after inoculation. Data are shown as mean $\pm$ SE for three replicates. *, Highly significant levels ($P < 0.05$, one-way analysis of variance) compared to day 0 cultures.

enhanced in the presence of IL-4. The up-regulation of CXCR4 RNA by IL-4 however, was species-specific because it was not observed in Pt cells. Interestingly, the constitutive abundance of the CCR5 message was higher than that of CXCR4 mRNA, and IL-4 did not alter the

Figure 5. Semiquantitative RT-PCR analyses of CXCR4 and CCR5 mRNAs. A representative RT-PCR analyses using 1 μ g of RNA from Rh and Pt MDMs cultured in the absence and presence of exogenous IL-4. Data represent the means $\pm$ standard deviations of CXCR4 cDNA/ β -actin cDNA and CCR5 cDNA/ β -actin cDNA values. Macrophage cultures from three animals of each species were inoculated with each of the three viruses and analyzed in triplicate.

expression of CCR5 mRNA in MDMs from either species of macaque.

MCP-1 RNA Is Expressed in Encephalitic Brains of Rhesus Macaques Infected with X4 Virus

It has already been established that lentiviral encephalitis caused by R5 viruses is usually associated with enhanced production of MCP-1 in the brain parenchyma^{27,41} as well as in the CSF.³⁰ In the present study, because exogenously added IL-4 and infection with X4 virus additively enhanced MCP-1 production in Rh MDM cultures, we asked whether the encephalitis caused by the X4 virus was also associated with a similar increased production of MCP-1 in the CNS and the CSF compartments. We examined brain tissue from macaques that were uninfected and others that were infected with SHIV_{89.6}P, respectively. Equal amounts of RNA from brain samples were spotted on a membrane using a slot blot and hybridized with a ³²P-labeled MCP-1 cDNA probe. As shown in Figure 6A, a constitutive level of MCP-1 RNA was expressed in the deep white matter of the uninfected macaque brain but the level was enhanced in animals that developed encephalitis. Figure 6B is a densitometric scan of the slot blot showing that this enhancement was approximately twofold. Northern blot analysis was used to confirm the specificity of the MCP-1 probe. As shown in Figure 6C, RNA from the deep white matter region of the brain in animal with encephalitis had twofold higher levels of MCP-1 RNA than the normal brains. The presence of MCP-1 in the brains of encephalitic animals was further confirmed by *in situ* hybridization. Sections of brain from one of two encephalitic animals used in this study showed that MCP-1-expressing cells were confined mainly to microglial nodules (Figure 7A). No MCP-1 signal was detected in the lesion when a MCP-1 sense riboprobe was used (Figure 7B). Immunohistochemical analysis confirmed that the majority of the cells in the nodules were macrophages (Figure 7C) and that these cells were productively infected with the virus (Figure 7D). Further comparison of MCP-1 content in the CSF of normal and encephalitic animals showed that the latter had increased levels of the chemokine compared to the former (Figure 7E).

Discussion

In the present study we explored a correlation between the tropism of primate lentiviruses for host macrophages and the production of MCP-1 by the infected cells. The fact that enhanced production of the chemokine was caused by infection with X4, X4/R5, and macrophage-tropic R5 and X4 viruses suggested strongly that linkage between the two phenomena contributes to neuropathological outcome. In the case of the R5 viruses that can cause primary lentiviral encephalitis, the ability of the agents to replicate productively in host macrophages, supplemented with the observation that the infection caused enhanced production of MCP-1 by the cells, provided the rationale for both the

Figure 6. **A:** Slot blot analysis of mRNA for MCP-1 in the deep white matter region of the brains from normal (N) and SHIV89:6P-infected macaques with encephalitis (E). The autoradiogram is representative of two separate experiments. **B:** Laser densitometric scanning of the autoradiogram from normal and infected macaques with encephalitis. **C:** Northern blot analysis of MCP-1 mRNA in the deep white matter from a normal Rh macaque and from a SHIV89:6P-infected macaque with encephalitis.

cellular tropism of the virus in the brain as well as a mechanism for promoting further recruitment of the viral host cells into the brain. The linkage between the two factors is probably of major importance in the neuropathogenesis of R5 virus infections. The fact that IL-4 had minimal effects in

Figure 7. **A:** *In situ* hybridization of brain lesions from the deep white matter region of a SHIV89:6P-infected macaque with encephalitis using a MCP-1 riboprobe showing positive hybridization signal in the microglial nodules. **B:** *In situ* hybridization of a representative area from the brain of the same macaque that was hybridized with an MCP-1 sense RNA probe. **C:** Immunohistochemistry for macrophage-specific marker, CD68, in the deep white matter of the same macaque. **D:** *In situ* hybridization of the brain lesion from the deep white matter of the macaque with CNS lesions using a riboprobe specific for SIVgag RNA showing virus-positive cells in the microglial nodule. **E:** MCP-1 levels in the CSF of normal and SHIV89:6P-infected encephalitic Rh macaques. Original magnifications: $\times 140$ (A, C, D); $\times 70$ (B).

causing enhancement either of R5 virus replication or in production of MCP-1 in macrophages infected with these viruses suggested that this cytokine may not be absolutely essential for neuropathogenesis caused by the R5 agents. However, the finding that IL-4 did cause an increase in the constitutive level of MCP-1 production in uninfected macrophages, and that this cytokine is present in encephalitic brains,² suggests that it could indirectly promote development of the neuropathological syndrome by causing a global up-regulation of production of MCP-1 in the brain.

In contrast to the infections caused by R5 agents, the new data have provided a further understanding of the mechanism for the recently demonstrated neuropathogenic potential of X4 and X4/R5 SHIVs in Rh macaques. Usually, infection in macaques with SHIV_{KU-2} and SHIV_{89.6}P results in loss of CD4⁺ T cells. However, diseases associated with enhanced virus replication in tissue macrophages developed only in infected animals that were also injected with *S. mansoni* eggs.¹ Because IL-4-producing cells were prominent among the produc-

tively infected macrophages in these animals, we surmised that the cytokine had a role in enhancing virus replication in macrophages.¹ The present study in cultured macrophages showed the IL-4-mediated enhancement of virus replication was confined to infection of Rh macrophages caused by X4 and X4/R5 but not R5 viruses. Furthermore, this effect was accompanied by a selective enhancement of production of MCP-1 in X4 and X4/R5 virus-infected cells. The mechanism of IL-4-mediated up-regulation of X4 and X4/R5 virus replication in Rh macrophages was because of the selective enhancement of CXCR4 mRNA by IL-4. This effect was specific for Rh and not Pt MDMs, because virus production in Pt MDMs was not significantly affected by exogenous IL-4. A likely explanation for the lack of Pt cells to respond to IL-4-mediated enhancement of X4 and X4/R5 virus replication can be attributed to the combined effects of a lower abundance of CXCR4 mRNA on Pt cells and the failure of IL-4 to cause enhancement of CXCR4 mRNA, thereby making these cells innately resistance to infection by X4 and X4/R5 viruses. In contrast, the failure of enhancement of CCR5 virus replication in the presence of IL-4 could be explained by the inability of IL-4 to modulate CCR5 expression. It is of interest to note that the level of IL-4 receptor RNA expression in MDMs from both species was identical and remained unaffected with exogenous IL-4 (data not shown). Although IL-4 failed to cause enhanced replication of X4 and X4/R5 viruses in Pt macrophages, exogenous IL-4 did enhance MCP-1 synthesis in these cells. Thus, IL-4-mediated dual effects involving enhancement of X4 and X4/R5 virus replication and MCP-1 protein expression probably occurred through independent mechanisms.

Because MCP-1 is constitutively produced in normal brain, it is possible that the development of encephalitis in Rh macaques caused by the X4 viruses was contingent on productive replication of this type of virus in macrophages in the brain accompanied by a certain threshold level of MCP-1 in the brain. This threshold could have been achieved by primary infection with the R5 viruses in brain macrophages, but in the case of X4 virus infections, the achievement of the threshold may have required both replication of the virus and supplemental IL-4. Presumably, the opportunistic pathogens in the brain may have had a role in inducing the IL-4-producing cells. In Pt macaques, a review of more than 25 cases of SHIV infection in our laboratory had shown that the animals had developed full-blown AIDS but not encephalitis. This may have been a reflection of the innate resistance of macrophages from this species of macaques to support X4 virus replication.

In summary, MCP-1 has recently been identified as one of the chemokines that is present at high concentrations in the brains and CSF of individuals with lentiviral encephalopathy.^{27,30} Data presented here showed that production of the chemokine was enhanced in macrophages that were infected with either X4, X4/R5, or R5 lentiviruses, and in the Rh macrophages its production was even further increased after infection with the X4 and X4/R5 viruses and treatment with IL-4. Because IL-4 by itself caused enhancement of production of MCP-1 in

uninfected macrophages, beyond normal constitutive levels, it is evident that both MCP-1 and IL-4 are key players in the neuropathogenic outcome of lentiviral infections. Specifically, production of MCP-1 was very prominent in brain lesions populated by macrophages, and virus replication was greatly enhanced in these cells. These findings support the concept that the chemokine does indeed promote macrophage infiltration into the lesions and that this process becomes enhanced by infection in the cells with macrophage-tropic virus. In the case of X4 virus infections, IL-4 enhanced both MCP-1 production and virus replication in the Rh macrophages. Strategies aimed at curtailing production of these two host factors may therefore be useful as therapeutic interventions against onset of the neurological syndrome.

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