Differential Regulation of CC Chemokine Gene Expression in Human Immunodeficiency Virus-Infected Myeloid Cells

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Received January 19, 1999; returned to author for revision March 23, 1999; accepted June 15, 1999

The importance of chemokine expression on HIV infection has been emphasized by the discovery that infection of CD4+ T cells by M-tropic strains of HIV-1 is antagonized by the chemokines RANTES, MIP-1α, and MIP-1β, which are natural ligands of CCR5, a major coreceptor for macrophagetropic (M-tropic) isolates of HIV-1. Similarly, the CCR2b ligands MCP-1 and MCP-3 inhibit productive infection of PBMCs by both CCR5- and CXCR4-dependent strains of HIV-1, suggesting that expression of the MCP-1 chemokine may affect HIV infection via signaling through the CCR2 receptor and subsequent desensitization of the CCR5 and/or CXCR4 signaling pathway. Given the major role played by chemokine receptors in HIV-1 fusion/entry and the regulatory effects of chemokines on HIV infection, we examined the pattern of chemokine gene expression in HIV-1-infected myeloid cells and in primary monocyte/macrophages. Chronic HIV-1 infection of U937 monocytic cells increased the expression of RANTES, MIP-1α, MIP-1β, and IL-8 chemokine genes, but strongly inhibited PMA/PHA- and TNFα-induced MCP-1 gene transcription. HIV-1-mediated inhibition of MCP-1 transcription and secretion was further confirmed in de novo HIV-1-infected U937 cells and correlated with a delay in HIV-1-induced NFκB binding to the MCP-1 promoter. The inhibition of MCP-1 gene expression may provide a mechanism by which HIV-1 escapes the early influence of chemokine expression in monocytic cells.

INTRODUCTION

Human immunodeficiency virus (HIV) infects a number of cell types, including CD4+ T cells (Gallo et al., 1984), monocytes (Gartner et al., 1986), and macrophages (Koenig et al., 1986), as well as bone marrow precursor cells (Stanley et al., 1992), both in vivo and in vitro. In contrast to HIV replication in T lymphocytes, which results in T cell death, infection of cells of the monocytic lineage produces high levels of virus in the absence of significant cytopathicity (Roy and Wainberg, 1988; McElrath et al., 1989), suggesting that infected monocytes/macrophages (M/M) may serve as a reservoir for HIV persistence and spread in vivo (Meltzer et al., 1990; Meltzer and Gendelman, 1992; Perno et al., 1997). Promonocytic cell lines such as U937 and THP-1 have been widely used as in vitro models to investigate HIV-1 infection of M/M, although these cells differ markedly from their in vivo counterparts in terms of their susceptibility to different viral strains (Schuitemaker et al., 1992a; b). U937 cells become susceptible to macrophage-tropic (M-tropic) HIV-1 after treatment with retinoic acid (RA) or phorbol 12-myristate 13-acetate (PMA), agents that induce myeloid differentiation and expression of CCR5, a major fusion/entry cofactor for M-tropic HIV-1 (Moriuchi et al., 1998).

HIV infection is initiated by viral envelope glycoprotein gp120 interaction with cell surface CD4, followed by association with a coreceptor that triggers fusion of viral and cellular membranes (Deng et al., 1996; Feng et al., 1996; Doms and Peiper, 1997). Most monocyte-tropic HIV-1 strains use CCR5 chemokine receptor to enter macrophages and primary T lymphocytes (Dragic et al., 1996; Raport et al., 1996; Alkhatib et al., 1996), whereas T-tropic HIV-1 strains use CXCR4 (fusin, LESTR, or HUM-STR) for primary CD4+ T lymphocytes and CD4+ T cell lines (Feng et al., 1996; Berson et al., 1996). In addition, other chemokine receptors support infection by one or more virus strains in vitro, including CCR2b and CCR3 (Doranz et al., 1996; Choe et al., 1996; Rucker et al., 1996). The predominant virus strains isolated early after infection from asymptomatic, HIV-positive individuals use CCR5 as coreceptor, while viruses that emerge later during the course of infection use CXCR4 either in place of or in addition to CCR5 (Berger et al., 1998).

While coreceptors play a critical role in supporting entry of HIV-1 into cells, coreceptors may also influence postentry events (Edinger et al., 1997; Chackerian et al., 1997). Interaction of soluble HIV-1 and SIV Env proteins with CCR5 and CXCR4 results in receptor signaling (Davis et al., 1997; Weissman et al., 1997). Coreceptor...
signaling (coupling, phosphorylation, and internalization) are not required for Env-mediated membrane fusion or virus infection of transformed cell lines, but receptor signaling mediated either by HIV-1 interaction or by binding of ligand could influence postentry events of virus replication in primary cells such as macrophages (Aramori et al., 1997).

Chemokines are small (8- to 12-kDa) chemoattractant proteins that constitute the natural ligands for chemokine receptors. The β-chemokines RANTES, MIP-1α, and MIP-1β interact with CCR5 (Raport et al., 1996) and the α-chemokine stromal-derived factor-1 (SDF-1) serves as a ligand for CXCR4 (Bleul et al., 1996). Consequently, these chemokines can block infection of HIV-1 strains that use CCR5 and CXCR4, respectively. For example, RANTES, MIP-1α, and MIP-1β inhibit infection of CCR5-expressing peripheral blood mononuclear cells (PBMCs) by macrophage-tropic virus, while SDF-1 blocks infection of CXCR4+ HeLa/CD4 cells by the T cell line-adapted HIV-1 LAI (Cocchi et al., 1995; Bleul et al., 1996; Oberlin et al., 1996). Given these inhibitory effects, increased chemokine production may function as part of a protective host immune response against HIV infection and disease progression (Cocchi et al., 1995). Thus, β-chemokine gene expression is strongly enhanced in lymph nodes of patients with HIV disease, indicating that cells recruited to HIV-infected lymph nodes are likely to interact with β-chemokines before exposure to virus, since these molecules direct mononuclear cell traffic to sites of inflammation (Tedla et al., 1996; Adams and Lloyd, 1997). However, conflicting results have reported different effects of β-chemokine expression on HIV-1 replication in MDM and tissue macrophages (Dragic et al., 1996; Simmons et al., 1997; Schmidtmayerova et al., 1996; Coffey et al., 1997).

Given the major role played by chemokine receptors in HIV-1 fusion/entry and the regulatory effects of chemokines on HIV-1 infection, we sought to examine the pattern of chemokine gene expression in HIV-1-infected monocytic U937 cells and primary monocyte-derived macrophages (MDM). In uninfected cells, PMA/PHA treatment induced expression of β-chemokines such as RANTES, MCP-1, and MIP-1α and β, as well as the α-chemokines IL-8 and γ-IP10. Chronic and de novo HIV-1 infection of U937 cells increased the expression of these chemokine genes, but strongly inhibited PMA/PHA- and TNFα-induced MCP-1 gene transcript levels and MCP-1 protein secretion. HIV infection of U937 resulted in a delayed induction of NF-κB binding to the MCP-1 promoter and correlated with the repression of PMA/PHA- and TNFα-induced MCP-1 transcription. The inhibition of MCP-1 gene expression suggests a mechanism by which HIV-1 may escape the early influence of chemokine expression in monocytic cells.

**RESULTS**

Differential regulation of chemokine gene expression in chronically infected monocytic cell lines

The aim of this study was to examine the effect of the HIV infection on chemokine gene expression in myeloid cell lines (U937), monocytes, and MDM. To determine the pattern of chemokine expression in monocytic cell lines, promonocytic U937 cells were stimulated with PMA/PHA and harvested at different times after stimulation. RNA extracted from these cells was then subjected to RNase protection assay using a CK5 human chemokine template of the RiboQuant Multi-Probe RPA kit (PharMingen, San Diego, CA). Arrows indicate the migrations of labeled fragments protected from RNase digestion and corresponding either to chemokine mRNA (RANTES, γ-IP10, MIP-1β, MIP-1α, MCP-1, and IL-8) or to a control gene mRNA (L32 and GAPDH).

![FIG. 1. Kinetics of chemokine induction in PMA/PHA-stimulated U937 and U9-11B cells. U937 promonocytic and chronically HIV-1-infected U9-11B cells were left unstimulated (lanes 1 and 8) or treated with PMA (50 ng/ml) and PHA (10 ng/ml) for 1, 2, 4, 6, 8, and 10 h (lanes 2–7 and 9–14). Total RNA extracted (5 μg) from these cells was subjected to RNase protection assay using a CK5 human chemokine template of the RiboQuant Multi-Probe RPA kit (PharMingen, San Diego, CA). Arrows indicate the migrations of labeled fragments protected from RNase digestion and corresponding either to chemokine mRNA (RANTES, γ-IP10, MIP-1β, MIP-1α, MCP-1, and IL-8) or to a control gene mRNA (L32 and GAPDH).](image-url)
expected, no mRNA corresponding to T-cell-restricted lymphotactin or I-309 genes was detected in U937 cells (data not shown).

The effect of HIV-1 infection on uninduced or PMA/PHA-induced expression of chemokine genes was examined initially using U9-IIIb cells chronically infected with the HIV-1 IIIB strain (Fig. 1, lanes 8–14). In uninduced U9-IIIb cells, RANTES and MIP-1α mRNA were upregulated compared to noninfected U937 cells (Fig. 1, compare lanes 1 and 8). Stimulation of U9-IIIb cells with PMA/PHA increased MIP-1α, MIP-1β, and IL-8 mRNA expression with the same kinetics as observed in U937 cells (Fig. 1, lanes 9–14); the induced levels of chemokine RNA were increased 3- to 4-fold in infected cells compared to uninfected cells, indicating that HIV infection enhanced chemokine gene expression. Surprisingly, in HIV-infected U9-IIIb cells, MCP-1 mRNA levels were reduced dramatically; after 8 h of PMA/PHA stimulation, a 15-fold decrease in MCP-1 mRNA was detected (Fig. 1, lanes 9–14). Similarly, only low-level expression of γ-IP10 was observed after PMA/PHA induction in chronically infected U9-IIIb cells.

Regulation of chemokine expression during de novo HIV infection of U937 cells

De novo infection of U937 cells was performed to determine the pattern of chemokine expression during the course of HIV-1 infection; the progress of infection was monitored by reverse transcriptase activity (Fig. 2A). At different times after infection, cells were treated with either PMA/PHA or TNFα for 8 h and then analyzed for chemokine RNA accumulation. De novo HIV infection of U937 cells differentially modulated chemokine RNA expression (Fig. 2B); in unstimulated cells, the level of MCP-1 RNA decreased during the course of infection, whereas constitutive RANTES expression was not affected by infection (Fig. 2B, lanes 1–5). PMA/PHA-induced MCP-1 transcription was inhibited significantly after 6 and 8 days of infection (Fig. 2B, compare lanes 6, 8, and 9). Residual mRNA levels could be due to MCP-1 expression in uninfected cells. MCP-1 transcript levels were restored by day 24; the apparent decrease was due to lower recovery of RNA from 24 day-infected cells, as indicated by the weak signals for L32 and GAPDH (Fig. 2B, lane 10). Quantification of MCP-1 mRNA levels, using L32 and GAPDH as internal controls, revealed a higher MCP-1/L32 mRNA ratio at day 24 than at day 8 (see Fig. 3A). In contrast to chronic infection, de novo infection of U937 cells resulted in a significant decrease in PMA/PHA-induced MIP-1α, MIP-1β, and RANTES mRNA (Fig. 2B, lanes 6–8). This downregulation presented the same transient characteristics as observed for the MCP-1 gene (Fig. 2B, compare lane 6 and lanes 7–10). A similar decrease in MCP-1 mRNA levels was obtained when HIV-infected cells were stimulated with TNF-α, although mRNA of other chemokines were barely detectable after 8 h of TNF-α stimulation (Fig. 2B, lanes 11–15). To confirm that this downregulation was solely due to HIV infection, U937 cells were cultured for 4, 6, and 8 days and stimulated or not with PMA/PHA or TNFα for 8 h. No differences in the constitutive or induced chemokine expression pattern were observed during this period, indicating that the downregulation shown at early time of infection was specific to HIV-1 (data not shown).

![Image](https://via.placeholder.com/150)
Downregulation of MCP-1 mRNA and protein levels in de novo infected U937 cells

Quantification of MCP-1 mRNA levels during HIV infection demonstrated that PMA/PHA-induced transcription of MCP-1 was decreased more than threefold after 6 days of infection compared to levels in infected cells (Fig. 3A). Furthermore, uninduced and TNF-α-induced levels of MCP-1 RNA were also subject to the same downregulation, although the effect was less marked (Fig. 3A). To assess whether the amount of MCP-1 protein was similarly altered in HIV-infected U937 cells, secreted MCP-1 was measured by ELISA in supernatants of HIV-1-infected cells. Concomitant with the decrease in MCP-1 mRNA, the amount of MCP-1 secreted by PMA/PHA-induced cells decreased throughout HIV infection; by day 8, MCP-1 levels were reduced sixfold compared to uninfected cells (Fig. 3B). Interestingly, the decreased MCP-1 levels were nearly identical to those measured in untreated 8 day-infected cells, indicating that HIV infection completely abolished the capacity of U937 cells to secrete MCP-1 in response to PMA/PHA stimulation. Secretion of MCP-1 by uninfected and TNF-α-treated cells was also inhibited by HIV infection until day 8 (Fig. 3B). By 24 days of infection, the levels of MCP-1 protein were partially restored, as seen at the mRNA level (Figs. 3A and 3B). Quantification of RANTES mRNA levels and measurement of secreted RANTES showed a modest decrease (around 50%) of mRNA and a dramatic decrease of RANTES secretion in early times—days 4 and 6—of HIV infection in PMA/PHA- and TNF-α-stimulated cells (data not shown).

MCP-1 transcription is downregulated after in vitro differentiation of primary monocytes

Primary monocytes/macrophages were isolated from peripheral blood of uninfected individuals and examined for PMA/PHA-induced chemokine expression. The expression pattern in primary monocytes was almost identical to that observed in U937 cells (Fig. 4A, lanes 3 and 5). These data indicated that U937 cells reflected the characteristics of in vivo primary monocytes, at least in terms of the regulation of chemokine expression. This observation was confirmed by the marked differences between primary monocytes and nonadherent lymphoid cells (Fig. 4A, compare lanes 2 and 5). In contrast to unstimulated monocytes, which only weakly expressed chemokine RNA in the absence of stimulation (Fig. 4A, lane 4), constitutive transcription of RANTES, γIP-10, MIP-1α and β, MCP-1, and IL-8 was observed in lymphoid cells (Fig. 4A, lane 1). Furthermore, significant expression of lymphotactin—a T-cell-specific chemokine—was detected; also PMA/PHA stimulation of lymphoid cells slightly enhanced the mRNA levels of MIP-1α and β, but reduced that of MCP-1 (Fig. 4A, compare lanes 1 and 2).

In vitro culture of primary monocytes resulted in the downregulation of PMA/PHA-induced MCP-1 mRNA levels at 3, 6, and 9 days after seeding, but did not significantly affect expression of MIP-1α, MIP-1β, and IL-8 genes (Fig. 4, lanes 6 and 7). Interestingly, a significant downregulation of RANTES expression was also observed in primary monocytes after 3, 6, and 9 days of in vitro differentiation (Fig. 4, lanes 4–7). These data indicated that expression of MCP-1 RNA were downregulated when primary monocytes were differentiated into
macrophages. A similar observation was made when U937 cells were infected by the HIV-1 IIIB strain for the same period of time (data not shown). The similarities in MCP-1 and RANTES inhibition observed after HIV infection or after monocytic differentiation suggest a common mechanism resulting in MCP-1- and RANTES-specific repression.

Effect of antioxidant agents on chemokine expression in monocytic lineages

Chronic infection of myeloid U937 and PLB985 cells leads to constitutive NF-κB activity, due to enhanced IκBα turnover and increased NF-κB induction (Roulston et al., 1992; 1993; DeLuca et al., 1996). To assess the potential role of NF-κB in the coordinated regulation of chemokine genes, cells were treated with N-acetylcysteine (NAC) and pyrrolidinedithiocarbamate (PDTC)—antioxidant agents that specifically inhibit the NF-κB induction in a variety of cell types (DeLuca et al., 1998; Lee et al., 1997). NAC and PDTC treatment of U937 cells inhibited PMA/PHA-induced transcription of RANTES, MIP-1α, MIP-1β, and MCP-1 and reduced IL-8 gene expression (Fig. 5, lanes 4–6 of the corresponding panels). However, PMA/PHA-induced expression of MIP-1α and IL-8 was less sensitive to NAC or PDTC treatment in U9-IIIB cells than in uninfected U937 cells (Fig. 5, compare lanes 4–6 to 10–12 in each panel). Since NAC and PDTC have been shown to inhibit the induction of NF-κB by PMA, the upregulation of MIP-1α and IL-8-induced transcription in chronically infected U9-IIIB cells cannot be explained by sustained NF-κB activity in these cells. As expected, no effect of NAC or PDTC treatment was observed on MCP-1 and γIP10 expression in U9-IIIB cells. Similar results were obtained after stimulation of uninfected and HIV-infected cells by TNFα (data not shown). The inhibition of PMA/PHA-induced transcription of MCP-1 gene expression in U937 cells by NAC and PDTC treatment strongly suggests that activation of NF-κB activity is responsible for induced expression of these two genes and that chronic or de novo HIV infection may lead to decreased or inhibited NF-κB binding activity in U937 cells.

NF-κB binding to the A1-κB site of the MCP-1 promoter is inhibited by HIV

The MCP-1 promoter contains in its distal region two NF-κB sites—A1 and A2, located at −2645 to −2626 and −2264 to −2262.
NF-κB complexes

-2616 to -2597, respectively—which are involved in TPA-induced expression of MCP-1 (Ueda et al., 1997).

NF-κB binding to the MCP-1 promoter was assessed in HIV-infected U937 cells by EMSA using the A1-κB site of the MCP-1 promoter as probe. In control U937 extracts, two weak NF-κB complexes were detected which reacted with anti-p50 antibody (Figs. 6A, lane 1, and 6B, lanes 1–5). Stimulation of U937 cells with PMA/PHA or TNFα resulted in an approximately 10-fold induction of the two complexes (Fig. 6A, lanes 1, 6, and 11). Complex formation was blocked with anti-p50 antibody and was weakly inhibited with anti-p65 and anti-c-Rel (Fig. 6B, lanes 11–15 and 16–20). Thus the A1-κB site participates in PMA/PHA- and TNFα-induced expression of the MCP-1 gene, in agreement with data showing that this site of MCP-1 promoter is required for its TPA-induced expression (Ueda et al., 1997).

HIV infection of U937 cells resulted in the appearance of a slower migrating protein–DNA complex, which was further identified by supershift analyses as the p65–p50 complex (Figs. 6A, lanes 2–5, and 6B, lanes 6–10). Interestingly, stimulation of HIV-infected U937 cells with PMA/PHA and TNFα revealed a delay in the appearance of the p50–p65 complex early after infection—days 4 and 6 (compare Fig. 6B, lanes 2 and 3, with lanes 7, 8, 12, and 13) and a complete inhibition of the PMA/PHA- or TNFα-induced NF-κB complexes identified in control cells (Fig. 6A, lanes 6–8 and 11–13). At later times—8 or 24 days—the HIV-induced NF-κB complex was detected, but not the PMA/PHA- or TNFα-induced complexes observed in uninfected cells (Fig. 6A, lanes 9–10 and 14–15). Both the delayed induction of the HIV-specific NF-κB complex and the inhibition of the PMA/PHA- and TNFα-induced NF-κB activity correlated with the inhibition of PMA/PHA- and TNFα-induced expression of MCP-1 mRNA observed after 4 and 6 days of HIV-1 infection (Figs. 2B and 3A). An identical pattern of inhibition of NF-κB activation and binding was observed when the A2-κB site of the MCP-1 distal promoter was used as a probe (data not shown), indicating the involvement of both sites in MCP-1 regulation.

Inhibition of NF-κB binding to the RANTES promoter

To determine if the RANTES promoter may be similarly regulated by HIV-induced inhibition of NF-κB binding at early times after HIV-1 infection, NF-κB activity was assessed using the -43 to -30 region of the RANTES promoter, which contains two adjacent NF-κB sites (Lin et al., 1999). A similar pattern of PMA/PHA- and TNFα-induced NF-κB binding was observed (Fig. 7, lanes 1, 6, and 11) and similar inhibitory effects of HIV infection on NF-κB protein–DNA complexes were also detected (Fig. 7, compare lanes 2 to 5 with lanes 7 to 10 and 12–15). This observation suggests that identical mechanisms of inhibition of NF-κB binding activity may be responsible for the decrease in PMA/PHA- and TNFα-induced RANTES mRNA expression shown after 4 and 6 days of HIV infection (Fig. 2B).

DISCUSSION

The importance of chemokine gene expression during HIV infection has been emphasized by the finding that infection of CD4+ T cells by M-tropic strains of HIV-1 is antagonized by the chemokines RANTES, MIP-1α, and MIP-1β, the natural ligands of CCR5 (Raport et al., 1996). However, conflicting data regarding the influence of β-chemokines on HIV-1 replication in MDM and tissue
Coffey et al., position of specific NF-κB shown to constitute a potential activator of RANTES expression are due to an HIV-mediated increase in that upregulation of constitutive and induced chemokine expression in PLB985 cells produces constitutive NF-κB activity and 1998). Chronic infection of myelomonocytic U937 or macrophages have been reported, with observations of enhancement, inhibition, or no effect (Schmidtmaierova et al., 1996; Dragic et al., 1996; Simmons et al., 1997; Coffey et al., 1997). The dichotomous effects of β-chemokines on HIV replication appear to depend on whether monocytes and MDM are exposed to chemokines before, simultaneously with, or after infection (Kelly et al., 1998). Moreover, enhanced HIV-1 replication following inhibition of endogenous β-chemokines by neutralizing antibodies has been reported (Kinter et al., 1996). The levels of chemokine expression and secretion therefore influence the ability of HIV-1 virus to replicate into target cells.

Using U937 monocytic cells and HIV-infected U937 cells as a model of chronic HIV infection, Kelly et al., reported an increased level of constitutive expression of the β-chemokines RANTES and MIP-1α (Kelly et al., 1998). Chronic infection of myelomonocytic U937 or PL8985 cells produces constitutive NF-κB activity and recent data have shown that chronic and de novo HIV infection of monocytic cells results in selective activation of NF-κB (p50/p65) via the activation of the IKKβ subunit of the recently identified IkBα kinase (IKK) complex (DeLuca et al., 1999; Asin et al., 1999). Since several chemokine genes are regulated in part by NF-κB, it is likely that upregulation of constitutive and induced chemokine expression are due to an HIV-mediated increase in NF-κB transactivation in chronically infected cells (reviewed in Roulston et al., 1995). Actually, NF-κB has been shown to constitute a potential activator of RANTES expression (Moriuchi et al., 1997) and to be responsible for the serum- and endotoxin-mediated induction of MIP-1α expression in macrophages (Grove and Plumb, 1993). Similarly, TNFα- and PMA-induced expression of the MCP-1 gene is suggested to be mainly due to NF-κB activation (Freter et al., 1996; Ping et al., 1996; Ueda et al., 1997).

In this paper, we examined chemokine gene expression during HIV infection of monocytic cell lines and primary monocytes/macrophages. PMA/PHA-induced transcription levels of MIP-1α and MIP1-β as well as IL-8 genes were increased in chronically HIV-infected U937 cells. The differential effect of HIV infection on chemokine expression was highlighted by the complete inhibition of PMA/PHA-induced transcription of MCP-1 and γ-IP10 in U9-11IB cells, suggesting that HIV may bypass normal PMA/PHA- or TNFα-mediated induction of certain chemokine genes, such as MCP-1 and to a lesser extent γ-IP10. The inhibition of MCP-1 expression was confirmed at both mRNA and protein levels, during the course of de novo infection in U937 cells. Interestingly, inhibition was only transient, with the maximum decrease reached at 6–8 days after infection. In contrast to chronic infection, de novo infection of U937 cells produced a similar transient downregulation of MIP-1α and MIP1-β transcription, RANTES expression was decreased, and PMA/PHA-induced transcription of the IL-8 gene was unaffected. These discrepancies between chronic and de novo infection of U937 may reflect the in vivo complexity of HIV infection, the state of monocytic maturation, and the differentiation state at which the infection occurs. Interestingly, the similarities in MCP-1 and RANTES transcriptional inhibition induced by either HIV infection or in vitro differentiation of monocytes suggest a common mechanism that leads to MCP-1- and RANTES-specific repression, at least in monocytic cells. Finally, our data may provide an explanation of the functional alterations in chemotaxis associated with HIV infection of myeloid cells (reviewed in Roulston et al., 1995).

By EMSA, we showed that HIV infection of U937 cells led, at early times after infection, to a delayed induction of the HIV-specific NF-κB binding complex, as well as to a complete inhibition of PMA/PHA- or TNFα-induced NF-κB binding activity to the NF-κB sites of the MCP-1 promoter. This decrease in DNA binding activity observed at 4 and 6 days after HIV-1 infection may thus explain the repression of PMA/PHA- and TNFα-induced MCP-1 transcription occurring at early times of infection. The EMSA data also suggest that identical mechanisms of NF-κB inhibition may be responsible for the decrease in PMA/PHA- and TNFα-induced RANTES expression at 4 and 6 days after HIV infection. Furthermore, our results associate the appearance of an HIV-specific NF-κB complex with the increase in MCP-1 and RANTES mRNA observed in unstimulated cells or at later times of infec-
tion (days 8 and 24). However, other mechanisms, such as posttranscriptional control, could be involved in virus-induced activation or repression of the MCP-1 and/or RANTES genes, since it has recently been shown that induced expression of RANTES by the respiratory syncytial virus (RSV) is mediated by increases in promoter activity as well as stabilization of RANTES mRNA (Koga et al., 1999).

Transient inhibition of MCP-1 expression in U937 cells following HIV infection may constitute a novel mechanism by which HIV-1 escapes the influence of MCP-1 expression and subsequent CCR2 binding on virus entry/fusion or postentry events. Similarly, RANTES expression is also inhibited by HIV, at least in acute infection. Interestingly, MCP-1 and RANTES are targeted by another unrelated virus—human cytomegalovirus (HCMV)—which encodes a homologue of a CC chemokine receptor gene (US28) that is capable of binding MCP-1 and RANTES (Gao and Murphy, 1994). Depletion of these chemokines from the medium of HCMV-infected cells was shown to be partially due to continuous internalization of extracellular chemokine by expression of the US28 protein at the cell surface (Bodaghi et al., 1998). The observation that MCP-1 accumulates in the cerebrospinal fluid of individuals with CMV encephalitis and recruits mononuclear cells (Bernasconi et al., 1996), thus affecting the ability of virus to persist and replicate in the brain, suggests that inhibiting MCP-1 and RANTES by US28 is crucial for CMV productive replication in the brain. Similarly, inhibition of chemokine secretion by HIV-1 may counteract the inhibitory effects of chemokines on viral infection. In contrast to HCMV, downregulation of MCP-1 by HIV-1 is due to transcriptional repression. Consequently, reexpression of MCP-1 during the course of HIV-1 infection of mononuclear cells may alter virus spread in monocytes/macrophages.

MCP-1 chemokine receptor CCR2 is implicated in HIV infection by virtue of its ability to support infection by some strains in vitro (Doranz et al., 1996; Rucker et al., 1996; He et al., 1997). Furthermore, a polymorphism in the CCR2 chemokine receptor in which Val 64 is replaced by Ile (CCR2-64I) has been associated with a 2- to 4-year delay in the progression to AIDS (Smith et al., 1997). The relation between MCP-1 and HIV infection was further reinforced by the discovery that the CCR2b ligands MCP-1 and MCP-3 inhibited productive infection of PBMCs by both the CCR5 and CXCR4 strains of HIV-1 (Frade et al., 1997; Schols et al., 1997). However, the basis for the protective effects of CCR2-64I polymorphism and MCP-1/MCP-3 expression is currently unclear since only a few HIV-1 strains use CCR2b to infect CD4+ cells (Doranz et al., 1996). Recently, Lee et al. hypothesized that heterologous desensitization of CCR5 and CXCR4 signaling by the CCR2 receptor provides a link that might explain both the in vivo effects of the CCR2 gene variant and the antiviral activity of CCR2 ligands (Lee et al., 1998). Heterologous desensitization of the RANTES response by MCP-1 was previously reported in human monocytic cells (Charo et al., 1994). HIV-1 env can signal through CCR5 and CXCR4 (Davis et al., 1997; Weissman et al., 1997), and coreceptor signaling, although not required for env-mediated fusion or entry, can influence postentry events of virus replication in primary cells (Aramori et al., 1997). Thus, MCP-1 expression may affect HIV infection via signaling through the CCR2 receptor and subsequent desensitization of the CCR5 and/or CXCR4 signaling pathway.

HIV-1 Tat protein induces CCR5 and CXCR4 expression in relation to Tat-enhanced infectivity of M- and T-tropic viruses, notably in monocytes/macrophages (Huang et al., 1998). Furthermore, Tat, which is known to be chemoattractant for monocytes, has recently been demonstrated to interact with β-chemokine receptor CCR2 and CCR3, but not with CCR5 and to induce Ca2+ fluxes in monocytes (Albini et al., 1998). Tat activation of CCR2 may stimulate the recruitment of chemokine expressing cells toward a productively infected cell, favoring the spread of HIV infection. These findings indicate that alterations in β-chemokine expression by HIV-1, and particularly of MCP-1 and RANTES, is essential for the establishment of HIV-1 infection.

MATERIALS AND METHODS

Cell culture and isolation of primary monocytes and lymphocytes

Myelomonoblastic U937 as well as U9-IIB cells, infected with HIV-1 strain IIB, were maintained in RPMI 1640 (GIBCO, Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO) and 10 μg/ml of gentamicin (Shering Canada, Pointe-Claire, Canada). Cells were stimulated with either 50 ng/ml of PMA (Sigma Chemical Co., St Louis, MO) and 100 ng/ml of PHA (Sigma) or 10 μg/ml of TNFα (R&D Systems, Minneapolis, MN). Peripheral blood mononuclear cells were obtained from HIV-negative, healthy donors, separated by Ficoll/Hypaque (Pharmacia, Upsalla, Sweden) centrifugation as described previously (Conti et al., 1997), and seeded on FBS-coated plastic tissue culture plates (Mosier, 1984). After an overnight incubation at 37°C in RPMI 1640 supplemented with 30% FBS, nonadherent cells, which mainly constitute lymphoid cells, were removed and cultured in 10% FBS RPMI 1640. Adherent cells were extensively washed and cultured 10% FBS RPMI 1640. At 1, 3, 6, and 9 days, adherent cells were recovered and analyzed. We refer to monocytes as cells processed 24 h after seeding, while 3-, 6-, and 9-day cultured adherent cells were considered macrophages.
HIV infection of U937 and reverse transcriptase assay

U937 cells were incubated with concentrated HIV-1 strain IIIB, a clone derived from the molecular HXB2D (Fisher et al., 1985) at a m.o.i. of 0.3 (viral concentration of 10⁶ PFU/ml) in a serum-free medium, for 2 h at 37°C. After virus exposure, the medium was replaced. Samples were obtained every 2 or 3 days for assay of reverse transcriptase (RT) activity. RT activity was determined by incubating 50 µl of cell supernatants (pre cleared by centrifugation at 3000 rpm for 30 min at 4°C) with a reaction mixture containing [³²P]dCTP (2 Ci/ml, Amer sham, Cleveland, OH) in Tris–HCl (pH 7.9) for 20 h at 37°C as described (Lee et al., 1987). Radiolabeled nucleotides were precipitated on GF/A Whatman filters by using cold 10% trichloroacetic acid and 95% ethanol. Incorporation activity was measured by liquid scintillation.

RNA extraction and RNase protection assay

Total RNA from U937 and U9-IIIB cells was extracted using RNeasy Mini kit (Qiagen, Valencia, CA). RNA from primary monocytes/macrophages was prepared as described (Ausubel et al., 1989). Briefly, cells were lysed in a guanidium thiocyanate homogenization buffer (4.0 M guanidium thiocyanate, 0.1 M Tris–HCl, pH 7.5, 1% β-mercaptoethanol) and the cell lysate was homogenized by passage through a syringe. Sodium lauryl sarcosinate (0.5%) was added and the suspension was centrifuged at 5000 rpm for 10 min at room temperature. The lysate was layered on a cushion of CsCl (5.7 M). RNA of higher density (>1.8 g/ml) than that of other cellular components was separated by centrifugation for 24 h at 35,000 rpm. The RNA pellet was washed, resuspended in TE/SDS, reprecipitated in ethanol, centrifuged, washed, dried, and resuspended in sterilized H₂O. Total RNA (5 µg) was subjected to RNase protection assay using a CK5 human chemokine template of the RiboQuant Multi-Probe RPA kit as recommended by the manufacturer (Pharmingen, San Diego, CA). Labeled fragments protected from RNase digestion and corresponding to MCP-1 mRNA were quantified using the NIH Image 1.60 software package. Values were normalized to the L32 (housekeeping gene) mRNA levels and plotted as MCP-1/L32 mRNA ratios. Similar results were obtained in three independent experiments when the MCP-1 mRNA signal was normalized according to the GAPDH mRNA.

ELISA detection assay

Concentration of secreted MCP-1 protein was determined from 50 µl of the supernatants of cell cultures (pre cleared by centrifugation at 3000 rpm for 30 min at 4°C) using the Human Monocyte Chemotactant Protein-1 (hMCP-1) ELISA kit (BioSource Int., Camarillo, U.S.A.) as described by the manufacturer.

EMSA

Whole cell extracts were prepared as previously described (Kwon et al., 1998) and 10 µg was subjected to EMSA by using 32P-labeled probes, DNA binding buffer (10 mM HEPES, pH 7.9, 2% glycerol (v/v), 40 mM KCl, 1 mM EDTA, pH 8.0, 0.2 mM MgCl₂, 1 mM DTT, 0.05 mM PMSF), 0.2% NP-40, 0.5 µg of BSA, and 1 µg of poly(dI: dC). Incubation was performed for 30 min at room temperature. Oligonucleotides used were (−2645 to −2626) A1-kB of MCP-1 promoter, 5’-GATCTGGAACTTC-CAAAAG-3’; (−2561 to −2597) A2-kB of MCP-1 promoter, 5’-AGAGTGGAAATTCACACTCA-3’; and (−43 to −30) NF-κB of RANTES promoter, 5’-ACTCCCCCTAGGGAGTGCCCTCCTA-3’. The resulting protein–DNA complexes were resolved on 5% polyacrylamide (37:5:1) TBE 0.25X gels and exposed for 16 h. To demonstrate the specificity of protein–DNA complex formation, a 100-fold molar excess of unlabeled oligonucleotide was added to the extracts before adding labeled probe. Supershift analysis was performed by preincubating 1 µl of anti-p65, anti-p50, and anti-c-Rel antibodies (Santa Cruz Biotechnology Inc.) with extracts and binding buffer for 30 min at 4°C before adding probe.

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