HIV-1 Vpr regulates expression of β chemokines in human primary lymphocytes and macrophages

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Abstract: The HIV-1 *vpr* gene encodes a 14-kDa virion-packaged protein that has been implicated in viral pathogenesis. Vpr exhibits profound effects on human primary cells influencing proliferation, differentiation, apoptosis, and cytokine production, in part through NF-κB-mediated transcription. NF-κB, a potent transcription factor, activates many proinflammatory cytokines/chemokines upon infection. Here, we analyzed the effect of extracellular Vpr as well as the virion-associated Vpr on β chemokines (MIP-1α, MIP-1β, and RANTES) production in human macrophages and primary lymphocytes (PBLs). Macrophages and PBLs exposed to HIV-1 *vpr*$^+$ viruses or to recombinant Vpr protein produced significantly less β chemokines compared with cells infected with HIV-1 *vpr*$^-$ viruses or irrelevant control protein (Gag)-exposed cells. These results suggest that a Vpr-mediated increase in virus replication could be in part through down-regulation of chemokine production. *J. Leukoc. Biol.* 68: 366–372; 2000.

Key Words: *HIV-1 · Vpr · β chemokine · macrophages · PBLs*

INTRODUCTION

HIV-1 Vpr is a unique nonstructural protein that is associated with the virus particle [1–3]. Recent studies have quantitated that Vpr packaged at a concentration of 14–18 molecules per virion [4]. A detectable level of Vpr protein is present in the plasma and cerebral spinal fluid (CSF) of human immunodeficiency virus (HIV)-1 infected patients, suggesting that cell-free and/or virus-free Vpr is present *in vivo* [5]. HIV-1-mediated cellular destruction of bystander $CD4^+$ and non-$CD4^+$ cells suggests that exposure of uninfected cells to viral proteins, such as virion-associated or cell-free Vpr, could mediate these effects [6, 7]. *vpr*, although dispensable for viral replication in T cell lines and activated peripheral blood mononuclear cells (PBMC) [8, 9], is required for efficient replication in primary monocytes/macrophages [10–12]. However, presence of Vpr or addition of extracellular Vpr significantly increases the virus replication in primary T cells and established T cell lines [5, 9–12]. Several possible roles have been suggested for Vpr in HIV-1 replication. Vpr transactivates HIV-1 long-terminal repeat (LTR) moderately and thus may upregulate viral gene expression in newly infected cells before the appearance of Tat [12, 13]. Furthermore, *vpr* regulates many host cellular events, such as cell-cycle arrest and cellular transcription, to increase virus production [14–16].

Chemokines are members of a family of related proinflammatory cytokines, which are part of the normal immune responses [17]. In the context of HIV-1 infection, they play an important role by controlling the infection [18]. Primary cells ($CD8^+$ and macrophages) are believed to play a critical role in controlling HIV infection by secreting one or more of the β chemokines RANTES (regulated on activation, normal T cell expressed and secreted), macrophage inflammatory protein (MIP)-1α, and MIP-1β [19, 20]. The exact mechanism of action of this suppression is mediated by blocking the binding between the chemokine receptors and viruses [21, 22]. Previously, we have demonstrated that Vpr inhibits T cell proliferation and blocks production of certain cytokines by human peripheral blood lymphocytes (PBLs) *in vitro* [16]. To further understand the effect of Vpr on other cellular functions, we analyzed the effect of Vpr on β chemokines and its role in viral replication. We demonstrate that HIV-1 Vpr significantly down-regulates the synthesis and secretion of β chemokines in macrophages and lymphocytes. These results indicate that Vpr-mediated down-regulation of HIV-1 suppressive factors could be one of the mechanisms by which Vpr upregulates the virus replication in target cells.

MATERIALS AND METHODS

Cells and plasmids

HeLa and RD cells, obtained from the American Type Culture Collection (ATCC; Rockville, MD), were grown in a monolayer at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium, containing 10% fetal bovine serum (FBS), 1% penicillin, 1% streptomycin, and 1% L-glutamine. Proviral constructs pNL43 R^+E^-, pNL43 R^-E^-, and Env expression plasmid were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (NIH AIDS RRRP; Bethesda, MD), contributed by Dr. Landau [23].

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Isolation of normal human primary PBLs

Blood from HIV-1-negative healthy donors was used to isolate PBLs by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) gradient centrifugation. PBMCs were collected from the interface, and PBLs were recovered in PBS washings following two rounds of adherence to plastic. The cells were further purified by negative selection with anti-CD14 magnetic beads (Miltenyi Biotec, Auburn, CA) to remove monocytes. The purified lymphocytes were resuspended in RPMI 1640, supplemented with 10% heat-inactivated fetal calf serum (FCS), stimulated with phytohemagglutinin (PHA; 5 μg/ml) for 2 days, and cultured in interleukin (IL)-2 (5 U/ml) containing medium.

Isolation of macrophages

Mononuclear cells were isolated from the HIV-1-negative healthy donors by Ficoll-Hypaque density-gradient centrifugation at 1500 *g* for 45 min. After removing the interface (PBMCs) with a Pasteur pipette, cells were transferred to a new tube and washed with plain RPMI media followed by centrifugation three times at 1500 *g* for 5 min each. After washing, cells were resuspended in 10% human serum in RPMI with 1% penicillin-streptomycin and 1% glutamine at a concentration of 2×10^6 per ml. The cells were incubated at 37°C in polysterene T-75 flasks for 5 days. After incubation, the cells were washed with RPMI three times to remove nonadherent cells. The adherent monocytes were detached with ethylenediaminetetraacetic acid (EDTA). The purity of the cell populations thus isolated was >98%, determined by immunofluorescence staining for CD14 and CD3 expression. The cells were incubated in 6-well plates at a density of 1×10^6 cells/ml in RPMI medium supplemented with 10% human serum [24].

Virus infection studies using HIV-1 *vpr*$^+$ and HIV-1 *vpr*$^-$ viruses

To assess the effect of Vpr as a virion-associated molecule, we have used amphotropic pseudotype viruses with and without Vpr. Pseudotype viruses were produced by cotransfecting RD cells with pEnv and pNL43.HSA.R$^+$E$^-$ or pNL43.HSA.R$^-$E$^-$. Cells (PBLs) were subsequently infected with HIV-1 *env*-complemented, *vpr*$^+$ or *vpr*$^-$ viruses. To infect macrophages, we have complemented pNL43.HSA.R$^+$E$^-$ or pNL43.HSA.R$^-$E$^-$ proviral DNA with pVSV-G-Env. RD cells were transfected with pVSV-G-Env with pNL43.HSA.R$^+$E$^-$ or pNL43.HSA.R$^-$E$^-$. Seventy-two hours posttransfection, supernatant was collected, concentrated, and assayed for virus production by measuring the p24 antigen released into the medium. PBLs (5×10^6) or macrophages (1×10^6) were infected with 10 pg of p24 antigen-equivalent viruses for 4 h, washed thoroughly with plain RPMI medium, and resuspended in the appropriate growth medium. Env-complemented pseudotype viruses undergo a single round of infection within the target cells, which allows study of the Vpr-mediated effects in the absence of viral-mediated killing.

Recombinant Vpr protein production and purification

HIV-1 *vpr* gene was PCR-amplified using primers Vpr (+) 5′ ACGGATCCATGGAACAAGCCCCAGA 3′ and Vpr (−) 5′ TGGATCTACTGGCTCCATT 3′, and cloned into pBlue Bak His vector (Clontech, Palo Alto, CA). Vpr-6X His tag expression plasmid and AcNPV vector were cotransfected into *Sf* 9 cells using the baculovirus-expression vector system (BaculoGold, PharMingen, San Diego, CA). The baculovirus-expressed recombinant protein was purified from *Sf* 9 cells at 72 h postinfection by using Talon metal-affinity resin (Clontech). The purity and specificity determined by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE) were 95%; this was confirmed further by western blot using rabbit polyclonal anti-Vpr antibody.

HIV-1 Vpr treatment

Highly purified (>95%) recombinant Vpr was used to study the effect of extracellular Vpr protein. To establish dose profiles, Vpr protein was used at 0, 0.1, 1, 10, and 100 pg/ml concentrations. For time-course experiments in PBLs and macrophages, Vpr (100 pg/ml) was incubated with cells for 0, 1, 2, 4, 6, 12, 24, and 48 h. Normal human PBLs and macrophages were treated with different concentrations of Vpr protein or control protein Gag (baculovirus-made Gag, NIH AIDS RRRP) for 6 h, washed with phosphate-buffered saline (PBS), and resuspended in RPMI medium with 10% FBS. Supernatant from the treated cells was collected every 24 h for subsequent measurement of β-chemokine secretion by enzyme-linked immunosorbent assay (ELISA). Negative controls included mock (PBS) and irrelevant protein (Gag) treatment.

Infection

Cells (macrophages and PBLs) were infected with pEnv-complemented pseudotype HIV-1 *vpr*$^+$ or HIV-1 *vpr*$^-$ viruses. This system has been used successfully by many investigators to study the effects of virion-associated gene products on host cells [25]. Purified PBLs and macrophages were infected with 10 pg of p24 antigen-equivalent viruses, a dose that we have tested previously. Four hours postinfection, cells were washed three times with PBS and resuspended in RPMI medium supplemented with IL-2. Samples were collected at 24 h intervals and stored at −20°C to assess β-chemokine secretion.

Quantitation of secreted chemokines by ELISA

Quantitation of chemokines present in the supernatant was done using capture ELISA. We measured MIP-1α, MIP-1β, and RANTES within the culture supernatants. The chemokine assay kits were purchased from R&D Systems (Minneapolis, MN), and the assay was performed according to the manufacturer's protocol. Briefly, supernatants from the infected cell cultures were added to the wells in triplicate at different dilutions and incubated at 37°C for 2–3 h, followed by washing and incubation with detection antibodies for 1 h. Bound antibodies were developed by the addition of 3′,3′,5′,5′-Tetramethylbenzidine (TMB) peroxidase substrate and detected at 450 nm in an ELISA plate reader.

Effect of Vpr as virion-associated molecule on chemokine mRNA synthesis in infected cells by RNase protection assay

Human PBMCs were infected with pNL43 R$^+$E$^-$ or pNL43 R$^-$E$^-$ viruses. Two days postinfection, cells were collected, and total RNA was isolated using RNAgent RNA Isolation kit (Promega, Madison, WI). After precipitation with isopropanol, the RNA was rinsed with 70% ethanol, air dried, and resuspended in diethyl pyrocarbonate (DEPC)-treated water. Total RNA was measured by absorption spectrophotometry at 260 nm. The A_{260}/A_{280} ratio averaged 1.7–1.8. RNase protection analysis was carried out with the use of RiboQuant Multi-Probe RNase Protection Assay System (PharMingen). Briefly, unlabeled sense RNA probes, encoding for lymphotactin (LTN), MIP-α, MIP-β, monocyte chemoattractant protein (MCP)-1, RANTES, IL-8, interferon (IF)-inducible protein (IP-10), I-309, mouse ribosomal protein 32 (L-32), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were used for the assay.

For synthesis of radiolabeled antisense RNA, the final reaction mixture (20 μl) contained 100 μCi [α-^{32}P] uridine 5′-triphosphate (UTP; 3000 Ci/mmol; NEN, Cambridge, MA); 10 μmol UTP; 500 μmol each guanosine 5′-triphosphate (GTP), adenosine 5′-triphosphate (ATP), and cytidine 5′-triphosphate (CTP); 10 μmol dithiothreitol; 1 × transcription buffer; 12 U RNasin; 8 U T7 polymerase; and an equimolar pool of linearized template. After 1 h at 37°C, the mixture was treated with DNase buffer (90 μl) and RQ1 RNase-free DNase for 30 min at 37°C, and the probes were purified by extractions with phenol-chloroform and chloroform, and precipitation with ethanol; and were dried under vacuum. Dried probes were dissolved (3×10^5 counts/min^1/μl^1) in hybridization buffer and added (2 μl; 3×10^5 counts/min^1/μl^1) to tubes containing experimental sample RNA (10 μg) dissolved in 8 μl hybridization buffer. The samples were heated at 80°C for 3 min and incubated at 56°C for 16 h. The single-stranded RNA was then digested by addition of a solution (100 μl) of RNase and RNase T1. After incubation (30 min at 37°C), the samples were treated with 18 μl of a mixture of proteinase-K (1 mg/ml), SDS (5%), and yeast tRNA (200 μg/ml). The samples were extracted with Tris-saturated phenol and chloroform: isoamyl alcohol and precipitation with ethanol. The samples were dissolved in 5 μl gel-loading buffer (80% formamide, 1 mM EDTA, pH 8.0, 0.1% bromophenol, and 0.1% xylene cyanol), heated to 90°C for 3 min, and subjected to electrophoresis in standard 5% acrylamide-8 M urea-sequencing gels. Dried gels were placed on XAR film (Eastman Kodak, Rochester, NY) with intensifying screens and were developed at 80°C for 18 h. The GAPDH mRNA was used as an internal standard to determine equivalent amounts of RNA used in parallel reactions. All experiments were repeated at

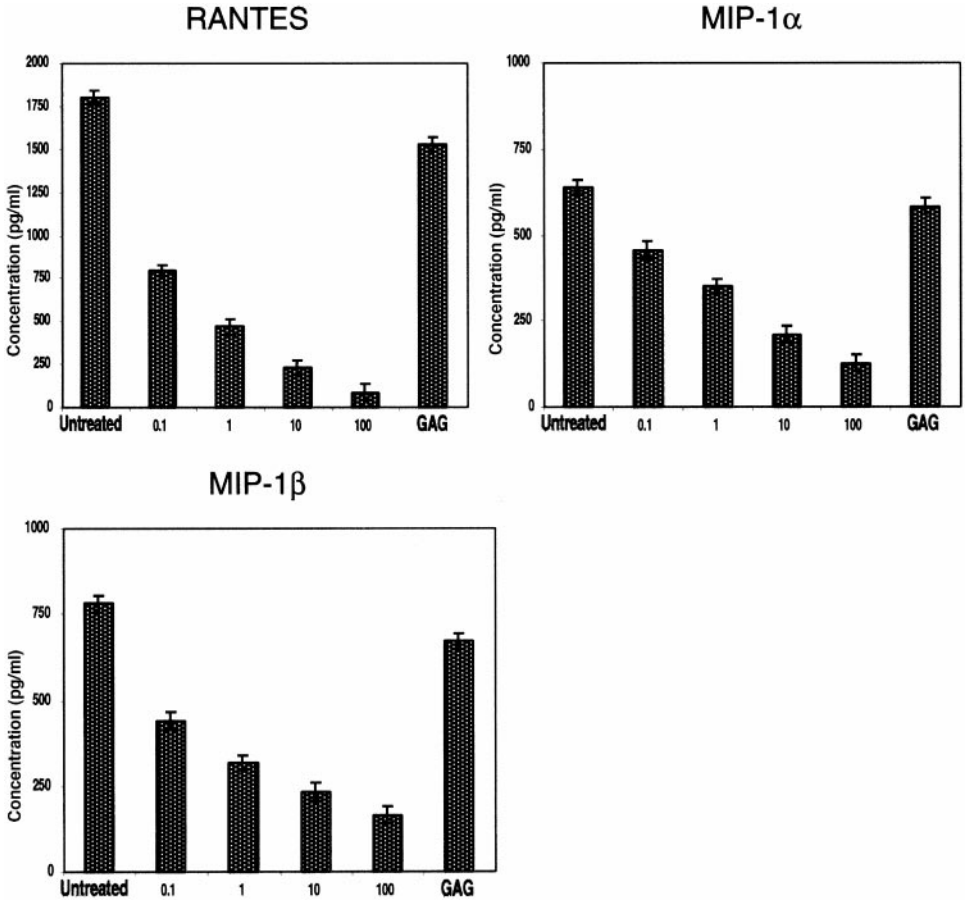

Fig. 1. Effect of rVpr protein on β-chemokine production in PBLs. Ficoll-Hypaque-fractionated human PBLs (1×10^6) were stimulated with PHA (5 μg/ml) and incubated in the presence of different concentrations (pg/ml) of pure rVpr protein or control protein (Gag). Cell-free supernatants were collected at every 24 h interval posttreatment and assayed for the release of β chemokines RANTES, MIP-1α, and MIP-1β in the medium. The mean of triplicate samples from one experiment is shown; these experiments were repeated multiple times, and similar results were obtained.

least two different times (24 and 48 h), using RNA isolated from at least two different experimental setups.

RESULTS

Effect of recombinant Vpr on chemokine production

To determine the effect of a transient exposure of Vpr protein on β-chemokine production in human primary cells, PHA-stimulated PBLs were treated with different concentrations of purified Vpr and assayed for production of the β chemokines MIP-1α, MIP-1β, and RANTES. The chemokine profiles are presented in **Figure 1**. Inhibition of chemokine production was significant at 100 pg/ml concentration, whereas no effect was observed at 0.1 pg/ml. Production of RANTES (20-fold), MIP-1α (4-fold), and MIP-1β (5-fold) was reduced in Vpr-treated cells at a concentration of 100 pg/ml, in comparison with control protein (Gag)-treated or -untreated cells. This suppressive effect was directly correlated with increasing Vpr concentration. For instance, MIP-1α concentration was 4- and 2.5-fold lower when Vpr was added at a concentration of 100 and 10 pg/ml, whereas almost no effect was seen when Vpr was added at 0.1 pg/ml concentration.

All experiments were conducted with highly purified recombinant Vpr protein. Yet, we considered the possibility that suppression could be a result of a contaminant in the Vpr preparations. To address this, we prepared a trypsin digest of Vpr and incubated with PBLs, because they were most sensitive to suppression of chemokine production by Vpr. No response was noted in these cells. To further confirm the specificity, the cellular response to Vpr was eliminated by immunodepletion with Vpr antisera but not with preimmune rabbit sera, confirming the specificity of Vpr protein effect (**Fig. 2**).

Effect of virion-associated Vpr protein on β-chemokine production

Vpr-mediated cellular functions, such as cell-cycle arrest and apoptosis, are mediated by Vpr protein introduced endogenously or Vpr delivered as virion-associated protein [25]. To further understand whether Vpr packaged in virions (in the absence of *de novo* protein synthesis) also influences the β-chemokine production, we infected human PBLs and macrophages with vpr^+ and vpr^- *env*-complemented pseudoviruses

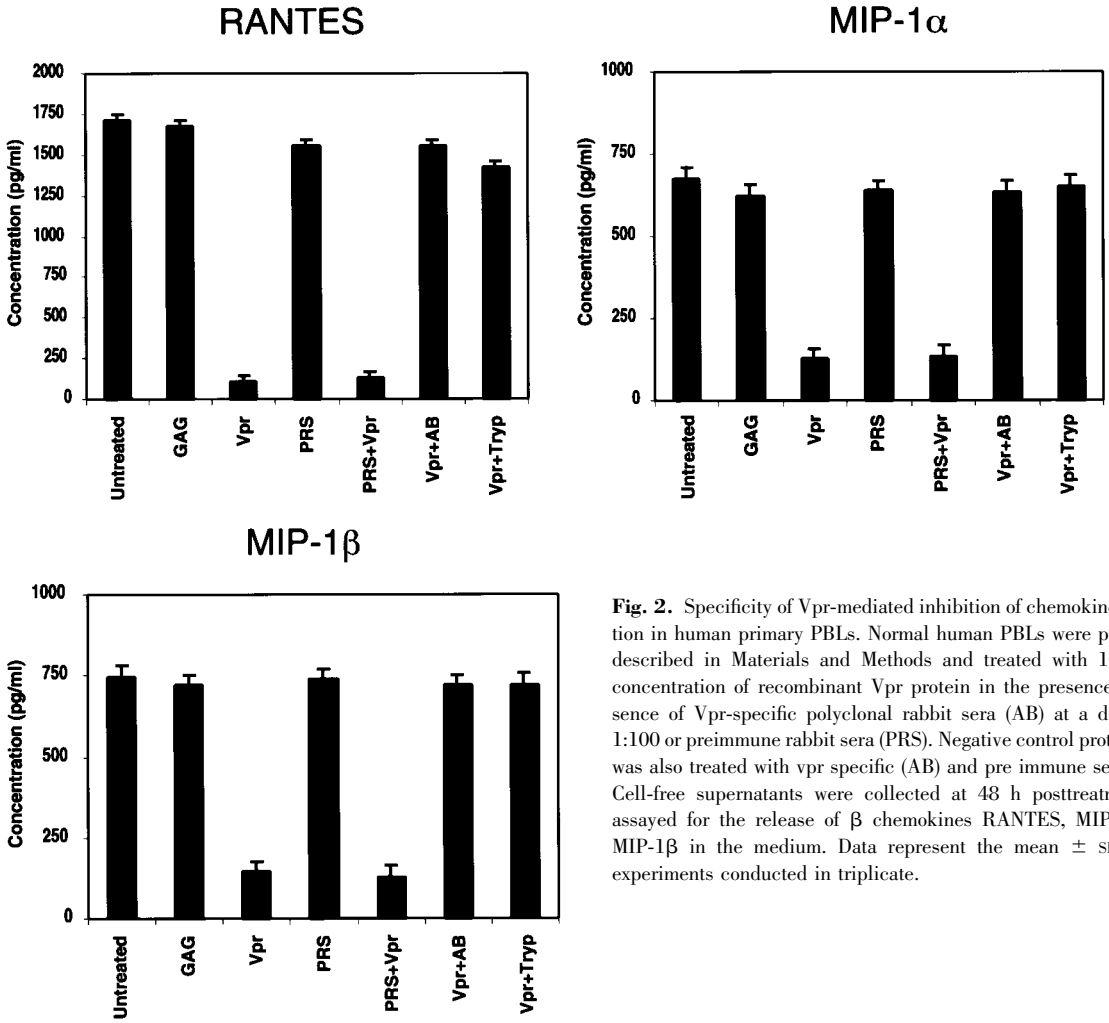

Fig. 2. Specificity of Vpr-mediated inhibition of chemokine production in human primary PBLs. Normal human PBLs were purified as described in Materials and Methods and treated with 100 pg/ml concentration of recombinant Vpr protein in the presence and absence of Vpr-specific polyclonal rabbit sera (AB) at a dilution of 1:100 or preimmune rabbit sera (PRS). Negative control protein (Gag) was also treated with vpr specific (AB) and pre immune sera (PRS). Cell-free supernatants were collected at 48 h posttreatment and assayed for the release of β chemokines RANTES, MIP-1α, and MIP-1β in the medium. Data represent the mean ± SD of four experiments conducted in triplicate.

and measured chemokine production. Results indicate that Vpr, provided as a virion-associated molecule in the presence of other viral proteins, inhibited chemokine production. PBLs infected with HIV-1 vpr^- virus produced 1633.9 ± 37.7 pg/ml (RANTES), 650.3 ± 34.3 pg/ml (MIP-1α), and 704.4 ± 28.9 pg/ml (MIP-1β), compared with 667.4 ± 33.6 pg/ml (RANTES), 241.8 ± 32.1 pg/ml (MIP-1α), and 302.7 ± 29.1 pg/ml (MIP-1β) produced by cells infected with vpr^+ virus (**Fig. 3A**). In a similar way, macrophages infected with Vpr^+ virus inhibited the production of RANTES (267.38±32.1 pg/ml), MIP-1α (321.12±33 pg/ml), and MIP-1β (421.11±33.2 pg/ml) when compared with virus infected with Vpr−, which produced 753.93 ± 32 pg/ml RANTES and 903 ± 32.14 pg/ml, and 845 ± 33.12 pg/ml for MIP-1α and MIP-1β, respectively (Fig. 3B). Cells infected with HIV-1 vpr^+ virus produced three- to four-fold lower levels of chemokines compared with *vpr*− virus-infected cells.

The changes in RANTES, MIP-1α, and MIP-1β secretion in the infected PBLs and macrophages peaked at 24–48 h after infection (unpublished results). The production of MIP-1α and MIP-1β was comparable in lymphocytes and macrophages. However, RANTES secretion in macrophages was lower than PBL's, because macrophages are not a significant source of RANTES production [26].

We next examined whether Vpr blocked the secretion of chemokine or inhibited the synthesis of chemokine mRNA. HIV-1 vpr^+ and vpr^- virus-infected cells (from the above experiments) were used as a source for RNA isolation. RNase protection assay was performed to examine specific mRNA levels. HIV-1 vpr^+ virus-infected cells show significantly lower amounts of messages for several β chemokines (including RANTES, MIP-1α, MIP-1β, IL-8, and I-309) compared with vpr^- virus-infected or -uninfected cells (**Fig. 4**), suggesting that Vpr affects the synthesis and secretion of chemokines by transcriptional control. Further analysis indicates that Vpr down-regulates chemoattractants for naive T cells and neutrophils (IL-8), CD8 T cells (MIP-1α and IL-10), monocytes (I-309), and CD4 memory T cells (RANTES). However, Ltn (lymphotactin), a chemoattractant for pre-T cells to the thymus, was not altered by Vpr.

DISCUSSION

HIV-1 Vpr, a 14-kDa virion-associated protein, functions during the early and late stages of the virus life cycle. It exerts its effect in two ways: (1) enhancing virus replication by efficient translocation of the viral preintegration complex

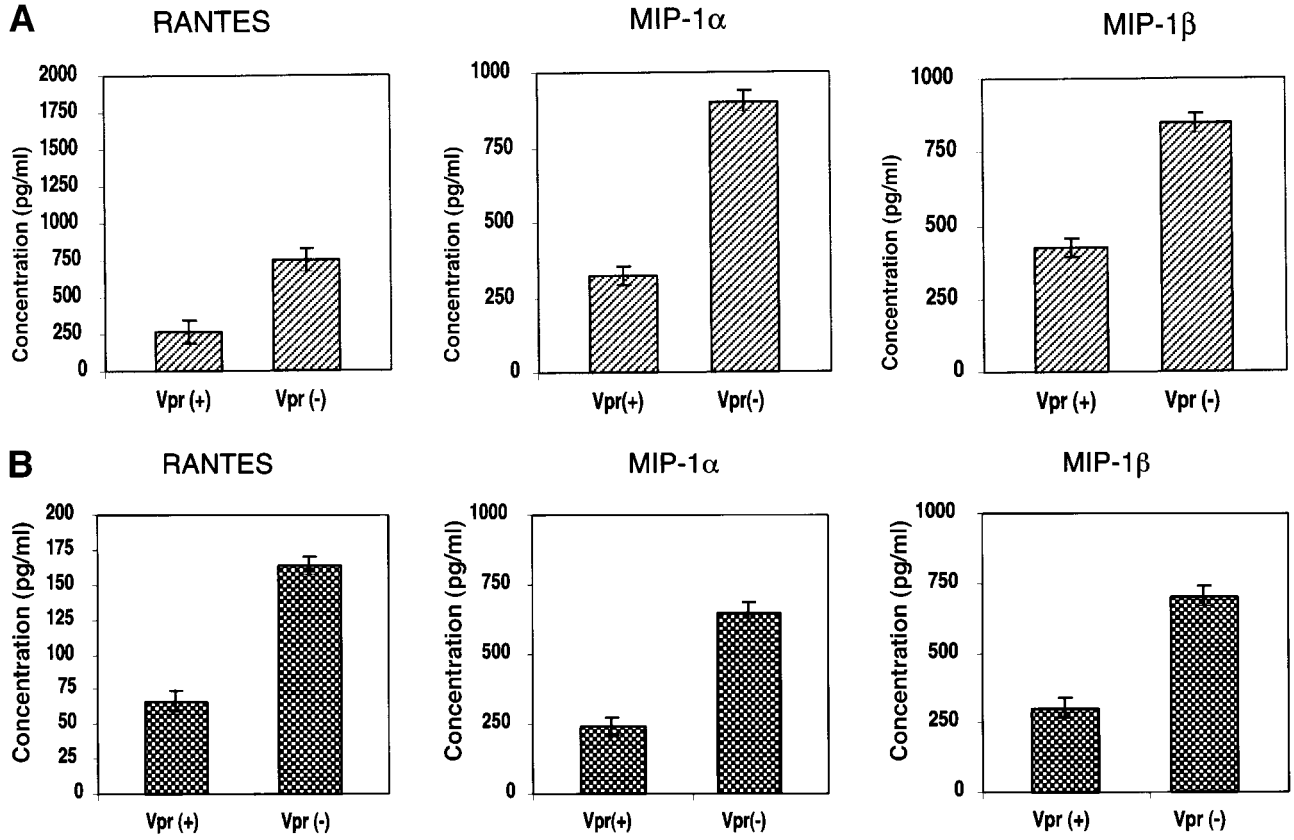

Fig. 3. (A) Effect of HIV-1 *vpr*$^+$ and *vpr*$^-$ virus on β-chemokine production on PBLs. Ficoll-Hypaque-fractionated normal human PBLs (1×10^6) were stimulated with PHA (5 μg/ml) and infected with HIV-1 R^+E^- and HIV-1 R^-E^- amphotropic pseudoviruses, as described in Materials and Methods. Cell-free supernatants were collected at 48 h posttreatment and assayed for the release of β chemokines RANTES, MIP-1α, and MIP-1β in the medium. The mean of triplicate samples from one experiment is shown; these experiments were repeated multiple times, and similar results were obtained. (B) Effect of HIV-1 *vpr*$^+$ and *vpr*$^-$ virus on β-chemokine production on macrophages. Purified human macrophages (1×10^6) were grown with RPMI medium supplemented with 10% human serum and infected with HIV-1 R^+E^- and HIV-1 R^-E^- pseudoviruses, complemented with pVSV-G Env as described in Materials and Methods. Cell-free supernatants were collected at every 24 h interval posttreatment and assayed for the release of β chemokines RANTES, MIP-1α, and MIP-1β in the medium. The mean of triplicate samples from one experiment is shown here. These experiments were repeated multiple times, and similar results were obtained.

and (2) modulating host cellular transcription to increase virus synthesis [14, 27, 28]. Many of the Vpr-mediated cellular events have been observed in a wide variety of cell lineages, suggesting that Vpr targets basic eukaryotic cellular pathways [29, 30]. Vpr, delivered as a recombinant protein, as a virion-associated molecule (in the absence of *de novo* protein synthesis), or as part of the viral genome, is capable of increasing the viral replication in human primary cells and established cell lines [7]. This suggests that contribution of HIV-1 *vpr* accessory gene product may play a role in host cellular dysregulation and disease progression [31].

On the cellular level, HIV-1 *vpr* exerts significant effects on cellular proliferation, differentiation, regulation of apoptosis, modulation of cytokine production, and suppression of host cell-mediated NF-κB transcription [16]. Primary lymphocytes ($CD4^+$) and macrophages/dentritic cells are the major reservoirs for HIV-1 infection [32, 33]. Interestingly, they are also the prime immune cells that play a significant role in immune activation upon infection. They release many proteins, namely, cytokines and chemokines, which are proinflammmatory and antiviral [34]. Chemokines are members of a family of related proinflammatory cytokines, which have a variety of biological properties, including chemotaxis and activation [35]. Chemokines are important particularly in the molecular regulation of leukocyte trafficking from the lymph nodes to the site of inflammation or infection. Activation of these factors is controlled mainly by NF-κB-mediated transactivation upon stimuli. Our results indicate that Vpr, provided as an extracellular protein or as a virion-associated molecule, is capable of down-regulating the synthesis and secretion of β chemokines. However, the exact mechanism by which Vpr inhibits chemokines synthesis/secretion is not completely understood. This could be a direct effect at the transcriptional level or an indirect effect by inhibiting the transcription factors such as NF-κB [16, 36]. Studies using mutant NF-κB binding sites and IκBα competition have shown that transcription factors, such as NF-κB and SP-1, are important for RANTES gene expression [37].

Chemokines, in addition to activating inflammatory responses, also have antiviral activity against HIV-1 through interfering with HIV-1 natural ligands [38, 39]. Previous studies have clearly shown that Vpr increases viral replica-

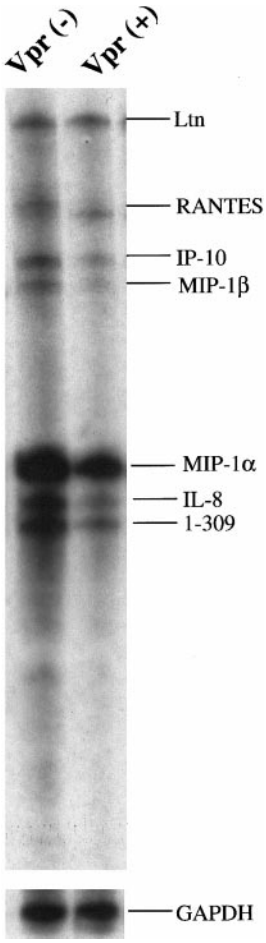

Fig. 4. Effect of HIV-1 vpr^+ and vpr^- virus on β-chemokine RNA in infected total PBMCs. Human PBMCs were infected with HIV-1 vpr^+ and HIV-1 vpr^- pseudotype viruses. Forty-eight hours postinfection, cells were collected, total RNA was isolated from the infected cells, and RNase protection assay was performed as described in Materials and Methods. Lane Vpr (−), PBMCs infected HIV-1 vpr^- viruses; lane Vpr (+), PBMCs infected with HIV-1 vpr^+ viruses.

tion *in vitro* and *in vivo* [5, 40, 41]. Results presented in Figures 1–3 demonstrate that Vpr as a viral protein or as a virion-associated molecule inhibits β-chemokine production in human primary cells, including human macrophages. Conversely, the virus production was also enhanced in the same culture (unpublished results). Based on our observation, it is also conceivable that Vpr could interfere with β-chemokine production to increase virus production. In conclusion, this study shows that one of the HIV-1 virion-associated accessory proteins, Vpr, could play a significant role in modulating the host immune system as well as influencing the viral replication to benefit the virus. Understanding the mechanism by which Vpr mediates these immune evasion strategies would open new avenues for the development of novel therapeutic agents.

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