relevant concentrations, AZTMP acts as a potent inhibitor of the transport of pyrimidine nucleotide sugars into the Golgi complex, thereby inhibiting protein glycosylation and altering glycosphingolipid synthesis (Yan et al., 1995). Therefore, AZTMP may elicit cytotoxic effects on rapidly growing erythrocytes and neutrophil precursors, both by interfering with nuclear DNA replication and by compromising the function of membrane receptors involved in receiving of extracellular stimuli required for cell growth and differentiation. From these observations it seems reasonable to speculate that either decrease in the intracellular concentration of AZTMP or compensatory mechanisms that improve the signal transduction for erythropoiesis and myelopoiesis mediated by cytokines contributed the recovery from hematological toxicities.

Two mechanisms may be related to the decrease in the concentration of AZTMP: altered metabolism of nucleoside analogues due to impaired nucleoside phosphorylation and increased efflux of the compounds by membrane transport mechanisms (Schuetz et al., 1999; Wijnholds et al., 2000). These mechanisms have been considered to contribute to the cellular drug-resistance (Dianzani et al., 1994; Groschel et al., 1997; Fridland et al., 2000; Turriziani et al., 2000). However, there was no evidence of treatment failure for patients in our PCV group as we found an increase in $CD4^+$ cell counts and an undetectable HIV-RNA load. Furthermore, the MCV level which is associated with the intracellular increase of AZTMP was kept high. These observations suggest that decrease in the level of AZTMP in the course of long-term treatment is unlikely although we must determine longitudinal changes of intracellular AZTMP level in precursors of blood cells in patients on Combivir treatment. Other compensatory mechanisms against the hematological toxicity may occur. An increase in erythropoietin or granulocyte-colony stimulating factor (G-CSF) levels in compensation for chronic anemia or neutropenia is another notion.

Acknowledgments

This work was supported in part by the Ministry of Health, Labor and Welfare of Japan (H-15-AIDS-001, -011, -015 and H-13-AIDS-001).

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研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Moriuchi M, and **Moriuchi H.**	Seminal fluid enhances replication of human T-cell leukemia virus type I: Implications for sexual transmission.	***J Virol***	78	12709-12711	2004
Moriuchi M, and **Moriuchi H.**	Cell-type-dependent effect of transforming growth factor-β, a major cytokine in breast milk, on human immunodeficiency virus type 1 infection of mammary epithelial MCF-7 cells or macrophages.	***J Virol***	78	13046-13052	2004

JOURNAL OF VIROLOGY, Nov. 2004, p. 12709–12711 Vol. 78, No. 22
0022-538X/04/$08.00+0 DOI: 10.1128/JVI.78.22.12709–12711.2004

Seminal Fluid Enhances Replication of Human T-Cell Leukemia Virus Type 1: Implications for Sexual Transmission

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Received 9 May 2004/Accepted 12 July 2004

Seminal fluid enhanced human T-cell leukemia virus type 1 (HTLV-1) infection by transactivating the HTLV-1 long terminal repeat promoter, which is chromosomally integrated in a cell-type-dependent manner. Our data may indicate a potential role for seminal fluid in the sexual transmission of HTLV-1 and imply complex features of regulation of HTLV-1 expression.

Human T-cell leukemia virus type 1 (HTLV-1), the causative agent of adult T-cell leukemia and HTLV-associated myelopathy, is transmitted vertically via breastfeeding and horizontally via sexual intercourse. Male-to-female transmission occurs exceedingly more frequently than female-to-male transmission (15). Given such a disproportion between genders in susceptibility to sexual transmission of HTLV-1, it is possible that a semen-derived factor(s) facilitates male-to-female transmission (10–12). We show here that seminal fluid enhances in vitro HTLV-1 infection. We also report that the seminal fluid-mediated effect on HTLV-1 expression requires its chromosomal integration and is cell type specific.

(This report was previously presented in part at the 11th International Conference on Human Retrovirology: HTLV and Related Viruses, 2003 [abstract P96].)

Seminal fluid enhances HTLV-1 replication and transmission. Seminal fluid was prepared from healthy male volunteers as described previously (1). Peripheral blood mononuclear cells (PBMC) obtained from asymptomatic HTLV-1 carriers were cultured in the presence or absence of seminal fluid.

HTLV-1 p19 antigen levels were measured as described previously (10). Cellular DNA was extracted by a QIAamp blood DNA kit (QIAGEN K.K., Tokyo, Japan) and subjected to PCR with a QuantiTect SYBR Green PCR kit (QIAGEN). The upstream and downstream primer sequences in the HTLV-1 *tax* gene that were selected for PCR analysis were 5′-CCCACTTCCCAGGGTTTGGACAGAG-3′ and 5′-CTG TAGAGCTGAGCCGATAACGCG-3′, respectively. Quantitative determination of the amplified products was done with the iCycler iQ Real-Time Detection System (Bio-Rad Laboratories, Inc., Hercules, Calif.). Heat activation (15 min at 95°C) of hot-start *Taq* polymerase was followed by 50 cycles of denaturation (30 s at 95°C), annealing (30 s at 50°C), and extension (30 s at 72°C). The cell numbers used for PCR analysis were confirmed by simultaneous PCR of the *tubulin* gene. Cellular RNA was extracted with a QIAamp RNA kit (QIAGEN) and subjected to PCR with a QuantiTect SYBR Green reverse transcription (RT)-PCR kit (QIAGEN). One-step RT-PCR was performed at 50°C for 30 min, followed by the same reactions as above. Tax mRNA levels were standardized by using those of tubulin mRNA.

HTLV-1 p19 antigen production and Tax mRNA levels were markedly enhanced by seminal fluid while proviral loads were modestly enhanced (Table 1). Although semen may be diluted in and poured out of the female genital tract after sexual intercourse, the concentration used in these experiments (1%) could be easily achieved in vivo. The viability of PBMC was found by trypan blue staining to be 80 to 90% and 85 to 95% in the presence and absence of 1% seminal fluid, respectively, throughout the experiments. These results suggest that seminal fluid can enhance HTLV-1 replication

To demonstrate whether seminal fluid can enhance viral transmission, PBMC from HTLV-1-uninfected individuals were cocultured with carriers' PBMC that had been treated with mitomycin C (MMC). Since MMC rendered the carriers' PBMC incapable of proliferating and supporting de novo

TABLE 1. Seminal fluid enhances replication and expression of HTLV-1[a]

Donor	Seminal fluid	Proviral load (copies/100 PBMC)	(Tax mRNA/tubulin mRNA) × 100	p19 antigen (pg/ml)
1	−	0.35	1.08	<25
	+	1.25	4.18	68
2	−	2.86	3.63	254
	+	10.6	42.5	2,880
3	−	0.12	0.76	<25
	+	0.19	9.24	186
4	−	1.02	2.20	45
	+	1.44	6.86	122
5	−	0.08	0.022	<25
	+	0.30	1.32	32
6	−	2.42	2.11	102
	+	4.41	10.1	654

[a] PBMC from six asymptomatic HTLV-1 carriers were propagated, and 3 million PBMC each were cultured in the presence or absence of seminal fluid (1%). Cell-free supernatants and cell pellets were collected on day 5. DNA and RNA were purified from the cell pellets, and real-time PCR and real-time RT-PCR were performed to estimate proviral loads and Tax mRNA levels, respectively. HTLV-1 p19 antigen levels in cell-free supernatants were determined by enzyme-linked immunosorbent assay.

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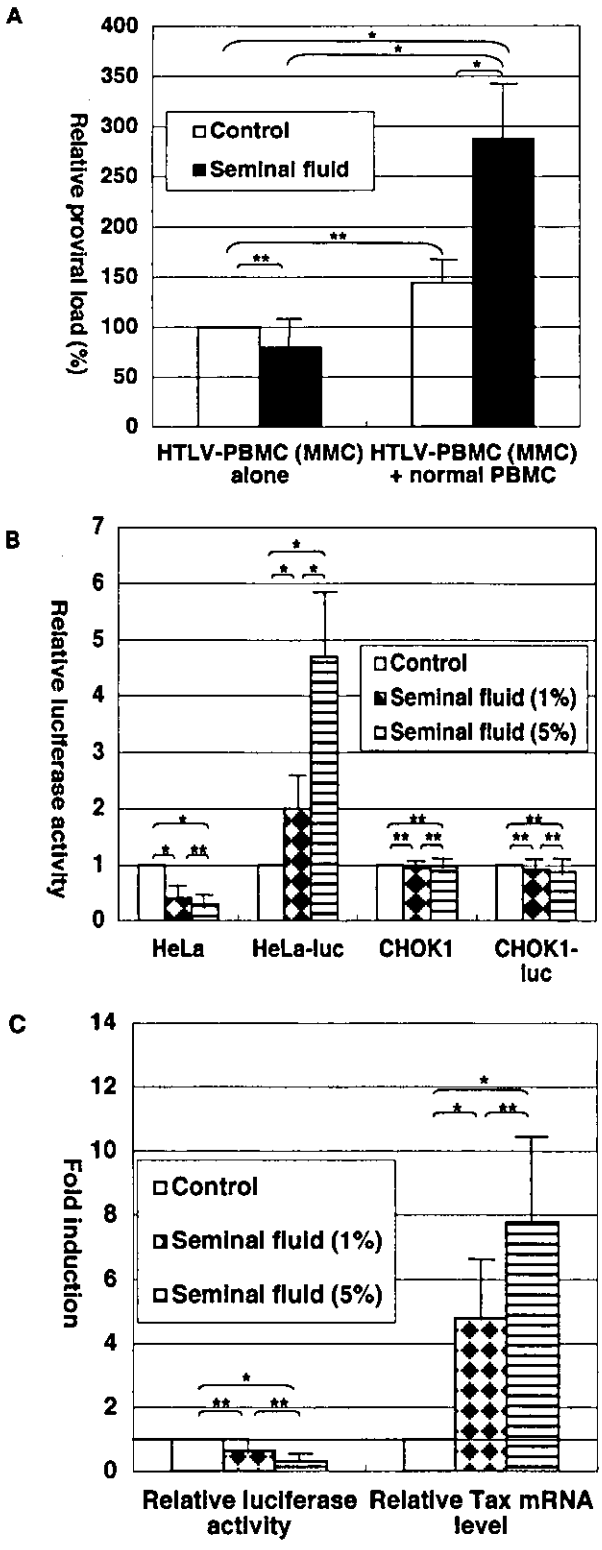

FIG. 1. Seminal fluid-mediated effects on HTLV-1 infection. (A) Seminal fluid facilitates de novo HTLV-1 infection. PBMC were isolated from three asymptomatic HTLV-1 carriers and treated with MMC as described previously (2). MMC-treated, HTLV-1-infected PBMC were cultured either alone or with PBMC derived from healthy uninfected donors at a ratio of 1:1. Where indicated, seminal fluid (1%) was added to the cultures. On day 7, whole cultures were harvested for DNA purification and proviral loads were determined by real-time PCR. Proviral loads in MMC-treated, HTLV-1-infected PBMC in the absence of seminal fluid were between 0.82 and 1.3 copies per 100 PBMC, and the results shown are means ± standard errors shown as proviral loads relative to them. Student *t* tests were performed for statistical significance (*, $P < 0.05$; **, $P \geq 0.05$). (B) Differential effects of seminal fluid on HTLV-1 transcription. HeLa and CHOK1 cells were transfected with pHTLV-luc and pMT-Tax, while HeLa-luc and CHOK1-luc cells were transfected with pMT-Tax alone. The transfected cells were left untreated or treated with the indicated concentrations of seminal fluid and harvested for luciferase assays 2 days after transfection. Data are means ± stand errors from six independent experiments, and results are shown as fold induction relative to the luciferase activity in untreated (control) cells. Student *t* tests were performed for statistical significance (*, $P < 0.05$; **, $P \geq 0.05$). (C) Seminal fluid increases expression from integrated provirus but decreases expression from transfected plasmid in PBMC. PBMC derived from asymptomatic HTLV-1 carriers were transfected with pHTLV-luc and either left untreated or treated with the indicated concentrations of seminal fluid. The transfected cells were harvested 2 days later, cell lysates were subjected to luciferase assays, and RNA purified from the cells was subjected to real-time RT-PCR for Tax and tubulin mRNAs. The data shown are means ± standard errors from six independent experiments, and results are shown as fold induction relative to those in untreated (control) cells. Student *t* tests were performed for statistical significance (*, $P < 0.05$; **, $P \geq 0.05$).

HTLV-1 infection, HTLV-1 replication in the coculture largely depends on viral transmission to PBMC from HTLV-1-uninfected individuals (4, 10). Seminal fluid increased HTLV-1-infected cell numbers in this coculture system but had little effect on infected cell numbers in MMC-treated PBMC alone (Fig. 1A), suggesting that seminal fluid facilitated de novo HTLV-1 infection in PBMC derived from uninfected donors.

Seminal fluid upregulates expression from the HTLV-1 LTR. Since we have previously demonstrated that certain seminal fluid-derived factors can transactivate the HTLV-1 long terminal repeat (LTR) (10–12), we investigated the effects of seminal fluid on the HTLV-1 LTR. Plasmid pHTLV-luc, provided by K.-T. Jeang (National Institute of Allergy and Infectious Diseases, Bethesda, Md.) (7), was transfected into PBMC with a Human T-Cell Nucleofector kit (Amaxa Biosystems) as described previously (13). Transfections of HeLa (cervical carcinoma) cells, CHOK1 cells, HeLa-luc cells, or CHOK1-luc cells (containing a chromosomally integrated HTLV-1 LTR-driven luciferase gene), also gifts of K.-T. Jeang (14), were performed by a modified calcium phosphate method (9). Transfection efficiency in these adherent cells was tested by cotransfection with pMACS14.1 (Miltenyi Biotec, Bergisch Gladbach, Germany), followed by flow cytometry for CD14 expression, and was found to be comparable (data not shown).

Unexpectedly, seminal fluid downregulated HTLV-1 LTR activity in HeLa cells (Fig. 1B). Interestingly, however, it enhanced HTLV-1 LTR activity in HeLa-luc cells (Fig. 1B), indicating that seminal fluid has opposing effects on the HTLV-1 LTR, depending on whether it exists episomally or is chromosomally integrated. The effects mediated by seminal fluid appear to be cell type dependent, because it had no effect on HTLV-1 LTR activity in CHO or CHO-luc cells (Fig. 1B). Trypan blue staining demonstrated that the seminal fluid concentrations used in these experiments were not toxic to those cell lines (data not shown). To clarify how seminal fluid influences HTLV-1 LTR activity in PBMC, we transfected carriers'

PBMC with pHTLV-luc and treated the transfected cells with seminal fluid. LTR activity from the episomal plasmid was determined by luciferase assay, and LTR activity from the integrated proviral DNA was inferred on the basis of Tax mRNA levels. As shown in Fig. 1C, seminal fluid downregulated the activity of the episomal HTLV-1 LTR while upregulating expression from the integrated HTLV-1 LTR. These results suggest that seminal fluid can enhance the transcriptional activation of proviral DNA in carriers' PBMC, probably contributing to seminal fluid-induced HTLV-1 replication and transmission.

Sexual, particularly male-to-female, transmission has been critical for the coexistence of HTLV-1 with the host because infected females subsequently transmit the virus to the next generation. Male-to-female transmission is exceedingly more efficient than female-to-male transmission, at least in part because this virus is highly cell associated (15), although involvement of cell-free virus in sexual transmission was not ruled out. Furthermore, male-to-female transmission may also be potentiated by the fact that the target tissue in the female genital tract is greater in size than that in the male genital tract.

This study suggests that seminal fluid-derived factors may play a role in sexual transmission. We have previously demonstrated that prostaglandin E2 (10), lactoferrin (11), and transforming growth factor β (12), all of which are major constituents of seminal fluid, could enhance in vitro HTLV-1 replication. However, while they upregulated HTLV-1 LTR activity in transient-expression assays (10–12), seminal fluid-mediated activity upregulated chromosomally integrated HTLV-1 LTR but not transiently transfected pHTLV-luc. Therefore, the effect of crude seminal fluid may not be simple addition of those factors but has more complex features. Our preliminary studies, including size fractionation and treatment with RNase A or proteinase K, indicate that not a single factor but a combination of several factors is involved in the effects of seminal fluid on the HTLV-1 LTR (data not shown).

Differential requirements for activation of the integrated and transiently transfected HTLV-1 LTR (14) and the human immunodeficiency virus type 1 LTR (3, 8) have been reported. The HeLa-luc cells used in this study were a pool of three independent HeLa clones with integration of two to four copies of pHTLV-luc, to minimize biases stemming from particular cellular integration sites (14). We also used PBMC from several different HTLV-1-infected donors to perform the experiments whose results are shown in Fig. 1B. Therefore, it is unlikely that the discrepancy in seminal fluid-mediated effects between the integrated and transiently transfected HTLV-1 LTR depends on integration sites.

Since seminal fluid-mediated transactivation of the HTLV-1 LTR was observed in PBMC and HeLa cells but not in CHOK cells, cell-type-specific mechanisms must be considered. Interestingly, induction of the expression of certain genes in cervical epithelial cells by seminal fluid has also been reported (5, 6). We confirmed this observation and extended the targets of this seminal fluid activity to PBMC. It is of note that seminal fluid induces expression of heat shock protein 70 (Hsp70) in cervical epithelial cells (5) and that HTLV-1 expression is enhanced following the cellular stress response that results in production of Hsp70 family proteins (2). Therefore, Hsp70 may play a role in the cell-type-dependent effect of seminal fluid on HTLV-1 infection.

Our preliminary studies suggested that the observed activity of seminal fluid on HTLV-1 LTR transactivation appears to result not from a single factor but from a combination of several factors. This complex feature of the effect of seminal fluid was not unexpected, considering the fact that seminal fluid contains a number of factors, including nucleases, proteases, and many other bioactive factors. Further studies are necessary to determine by what mechanisms and which factor(s) in seminal fluid mediates chromosomal integration-dependent transactivation of the HTLV-1 LTR.

We thank K.-T. Jeang for precious materials; S. Chiyoda, H. Okuda, and K. Deguchi (Nagasaki Red Cross Blood Center) for providing blood samples; and M. Yokoyama for excellent technical assistance.

This work was supported in part by grants provided by a Research for the Future Program (JSPS-RFTF97L00705) of the Japan Society for the Promotion of Science, The Japan Leukemia Research Foundation, The Mother and Child Health Foundation, and The ATL Prevention Program Nagasaki. This study was approved by the Institutional Review Board of the Nagasaki University School of Medicine.

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JOURNAL OF VIROLOGY, Dec. 2004, p. 13046–13052 · Vol. 78, No. 23
0022-538X/04/$08.00+0 DOI: 10.1128/JVI.78.23.13046–13052.2004

Cell-Type-Dependent Effect of Transforming Growth Factor β, a Major Cytokine in Breast Milk, on Human Immunodeficiency Virus Type 1 Infection of Mammary Epithelial MCF-7 Cells or Macrophages

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Received 9 May 2004/Accepted 6 July 2004

Breastfeeding plays a substantial role in mother-to-child transmission of human immunodeficiency virus type 1 (HIV-1). Mammary epithelial cells, as well as macrophages and lymphocytes, are thought to serve as sources of the virus in breast milk. Soluble factors in breast milk exert various biological functions, including immune tolerance or immune modulation, and may influence milk-borne infection with HIV-1. In this study we show that transforming growth factor β (TGF-β), a major cytokine in breast milk, inhibited HIV-1 infection of mammary epithelial MCF-7 cells but enhanced that of macrophages. TGF-β downregulated the HIV-1 long terminal repeat (LTR) promoter in MCF-7 cells but upregulated it in macrophages. Stimulation with TGF-β suppressed NF-κB binding to the HIV-1 LTR in MCF-7 cells, at least in part by downregulating induced IκB kinase expression. Cell type-dependent effects of TGF-β on HIV-1 expression may play a role in milk-borne infection with HIV-1.

Mother-to-child transmission accounts for the majority of human immunodeficiency virus type 1 (HIV-1) infections of children. In the developing countries where exclusive bottle-feeding is not feasible, up to 14 to 24% of babies born from HIV-1-infected mothers will be infected through breastfeeding (6, 7, 25). Although 75% of all milk-borne transmission appears to occur by 6 months of age, transmission continues throughout the lactation period (6, 7, 25).

HIV-1 strains are classified according to their coreceptor usage, and R5-tropic virus that utilizes CCR5 as an entry coreceptor is almost always a transmitting virus (31, 33). Breast milk contains both HIV-1-infected cells and cell-free virions, both of which appear to be contagious. The cell composition of breast milk changes throughout lactation period. Colostrum and early (<2 weeks after delivery) milk are rich in cells, such as macrophages (50 to 75%), neutrophils (20 to 40%) and lymphocytes (3 to 10%) (8), and macrophages appear to be the principal cellular carriers of HIV-1 in colostrum and early milk (28). Macrophages are susceptible to R5-tropic HIV-1 but much less to X4-tropic virus (1, 3) which utilizes CXCR4 as an entry coreceptor. Although the major source of HIV-1 in mature (>2 weeks after delivery) milk remains unknown, mammary epithelial cells, the predominant cell type in mature milk (8), have been shown to be susceptible to certain (mostly X4-tropic) HIV-1 strains (18, 28, 30).

Breast milk also contains a number of soluble factors that exert various functions, including antimicrobial activities and induction of immune tolerance or immune modulation. Certain milk whey components such as lactoferrin (24) and prostaglandin E (5, 29) have been shown to inhibit HIV-1 replication. Transforming growth factor-β (TGF-β) is a major cytokine in breast milk. Among the three isoforms of TGF-β, TGF-β1, expressed in endothelial, hematopoietic, and connective tissue cells, and TGF-β2, expressed in epithelial and neuronal cells, are contained in human milk. Colostrum contains 140 pg (67 to 186 pg) of TGF-β1 and 3,325 pg (1,376 to 5,394 pg) of TGF-β2 per ml, while mature milk contains 83 pg (17 to 114 pg) of TGF-β1 and 1,644 pg (592 to 2,697 pg) of TGF-β2 per ml (9).

TGF-β acts as an anti-HIV-1 or pro-HIV-1 factor, depending on cell type, virus strain, timing of treatment, or combination of other factors (13, 15, 17, 27); however, no substantial differences in effects on HIV infection among the TGF-β isoforms could be found (13). Here we studied whether TGF-β influences HIV-1 expression in major breast milk cells such as mammary epithelial cells and macrophages. Our data implicate bifunctional activity of TGF-β in mother-to-child transmission of HIV-1.

MATERIALS AND METHODS

Cells. Human mammary epithelial MCF-7 cells were obtained from the American Type Culture Collection and propagated in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco-BRL, Gaithersburg, Md.), 100 U of penicillin per ml, and 100 μg streptomycin per ml. Monocyte-derived macrophages were propagated from healthy donors' peripheral blood mononuclear cells by sorting CD14-positive cell fraction with AutoMACS (Miltenyi Biotec, Auburn, Calif.) according to the manufacturer's instructions and propagated in Dulbecco's minimal essential medium supplemented with 10% human male AB serum (Sigma Chemical Co., St. Louis, Mo.), penicillin and streptomycin. Purity of CD14-positive cells was more than 96% by flow cytometric analysis (data not shown). CD14-negative peripheral blood mononuclear cells, designated peripheral blood lymphocytes, contained less than 0.3% CD14-positive cells (data not shown).

Viruses and single- or multiple-round viral replication assays. For single-round viral replication assays, replication-incompetent luciferase reporter molecular clone pNL43-luc-R$^-$E$^-$ was complemented with Env glycoprotein from ADA (R5 HIV-1), JR-FL (R5 HIV-1), 89.6 (R5X4 HIV-1), ELI1 (primary X4

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HIV-1), HXB2 (X4 HIV-1) or amphotropic murine leukemia virus by transfecting 293T cells with plasmids encoding them, as described previously (23). Single-round viral replication assays were performed as described previously (23).

Multiple-round viral replication assays were performed with HIV-1 89.6 that was prepared by 293T cell transfection with the respective molecular clones, as described previously (21). Viral replication was assessed by reverse transcriptase assays for cell-free supernatants, as described previously (21).

Plasmids and transfection. Plasmid pGL-HIV-1-LTR contains the HIV-1 long terminal repeat (LTR) from strain HXB2 in pGL2-basic (Promega, Madison, Wis.) backbone. LTR sequence was truncated at −116 relative to transcription start site in pGL-HIV-1-LTR(−116), while NF-κB and SP1 binding sites on HIV-1 LTR were deleted in plasmids pGL-HIV-1-LTRΔNF-κB and pGL-HIV-1-LTRΔSP1, respectively (16). Plasmids pCMV-HA/Smad2 and pCMV-Flag/Smad3 were kindly provided by J. L. Wrana (Mount Sinai Hospital, Toronto, Canada) (11), and pCMV-hFAST1 was a gift of B. Vogelstein (Johns Hopkins Oncology Center, Baltimore, Md.) (32). Plasmid pHTLV-luc contains the human T-cell leukemia virus type I (HTLV-I) LTR promoter followed by the luciferase gene (10). One-day-old MCF-7 cell monolayer or 5-day-old monocyte-derived macrophages monolayer were transfected with modified calcium phosphate method, and luciferase assays were performed, as described previously (20, 22).

Reagents. Human lactoferrin were purchased from Sigma Chemical Co., and recombinant human TGF-β2 and polyclonal neutralizing anti-TGF-β antibody were purchased from R&D Systems (Minneapolis, Minn.). Early (<2weeks after delivery) and mature (>4 weeks after delivery) breast milk samples were donated from three healthy breastfeeding mothers. After centrifugation of the samples at 14,000 × *g* for 15 min, milk whey was separated from solid precipitate and stored at −80°C before use.

Nuclear extracts and gel mobility shift assays. Nuclear extracts were prepared from MCF-7 cells or monocyte-derived macrophages that had been untreated or treated with TGF-β (200 pg/ml), as described previously (19). Gel mobility shift assays were performed as described previously (19), with oligonucleotides corresponding to sequences spanning HIV-1 LTR NF-κB binding sites or SP1 binding sites as primers. Antibodies to NF-κB p50 or p65 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.).

Real-time RT-PCR. MCF-7 cells untreated or treated with TGF-β or milk whey were harvested, total RNA was extracted with a QIAamp RNA kit (Qiagen, Tokyo, Japan) and measurement of induced IκB kinase mRNA levels was performed by real-time RT-PCR with the iCycler iQ real-time detection system (Bio-Rad Laboratories, Inc., Hercules, Calif.) and QuantiTect SYBR Green RT-PCR kit (Qiagen). Primers were 5′-CCCAGCCCTACACGAAAGGACCTGC TTCTC-3′ and 5′-TCAGACATCAGGAGGTGCTGGGACTCTAT-3′. Reactions were incubated at 50°C for 30 min and then 95°C for 15 min, followed by 50 cycles of 95°C for 15 s and 62°C for 60 s. IκB kinase mRNA levels were normalized to tubulin mRNA by calculating the IκB kinase and tubulin ratio for each sample.

RESULTS

MCF-7 cells are susceptible to HIV-1 infection. It has been shown previously that primary mammary epithelial cells as well as certain breast cancer cell lines are susceptible to HIV-1. Therefore, we first tested whether MCF-7 cells are capable of supporting HIV-1 infection. To avoid any soluble factor derived from lymphocytes or macrophages and to precisely delineate susceptibility to the HIV-1 phenotypes, we performed single-round viral replication assays with HIV-1 molecular clone stocks prepared from 293T cell culture supernatants. As shown in Fig. 1A, MCF-7 cells supported infection with certain HIV-1 strains, although infection efficiency was far less than that of amphotropic murine leukemia virus. Compared to monocyte-derived macrophages that were more susceptible to R5 HIV-1 than to X4 HIV-1 (Fig. 1B) or peripheral blood lymphocytes that were susceptible comparably to R5 and X4 HIV-1 (Fig. 1C), MCF-7 cells apparently were more susceptible to dual (R5X4) or X4 HIV-1 than to R5 HIV-1. Since HIV-1 89.6 replicated comparably in all cell types tested, the following experiments were performed exclusively with this virus.

TGF-β inhibits HIV-1 infection of MCF-7 cells but enhances that of monocyte-derived macrophages. Next, we investigated

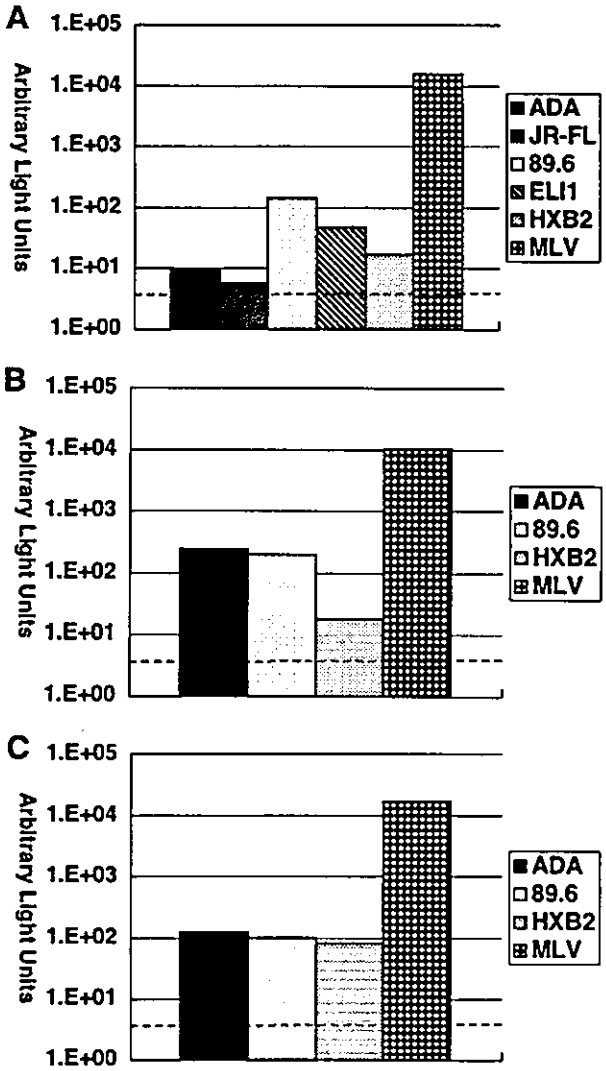

FIG. 1. Susceptibility of breast milk cell analogues to HIV-1 infection. In single-round viral replication assays, MCF-7 cells (A), monocyte-derived macrophages (B), and peripheral blood lymphocytes (C) were infected with NL4-3-Luc-R$^-$E$^-$ (3 × 10^5 cpm of reverse transcriptase activity each) supplemented with Env glycoprotein derived from the indicated strains, and the infected cell lysates were subjected to luciferase assays. A broken line indicates background luciferase activity. Representative results from three independent experiments are shown.

whether exposure to milk whey or its components can influence the susceptibility of mammary epithelial MCF-7 cells to HIV-1. While treatment of cells with crude milk whey or lactoferrin, a known anti-HIV-1 factor, suppressed HIV-1 infection, TGF-β had a minimal effect when added to MCF-7 cells prior to infection. In contrast, posttreatment with TGF-β as well as crude milk whey suppressed HIV-1 infection (Fig. 2A), suggesting that TGF-β has inhibitory effects on a postentry step(s) of the HIV-1 replicative cycle in MCF-7 cells. The inhibitory effect of crude milk whey posttreatment was partially restored by neutralizing anti-TGF-β antibody, suggesting that TGF-β played a role in that effect.

We also investigated the effects of milk whey or TGF-β on HIV-1 infection of macrophages, another major source of HIV-1

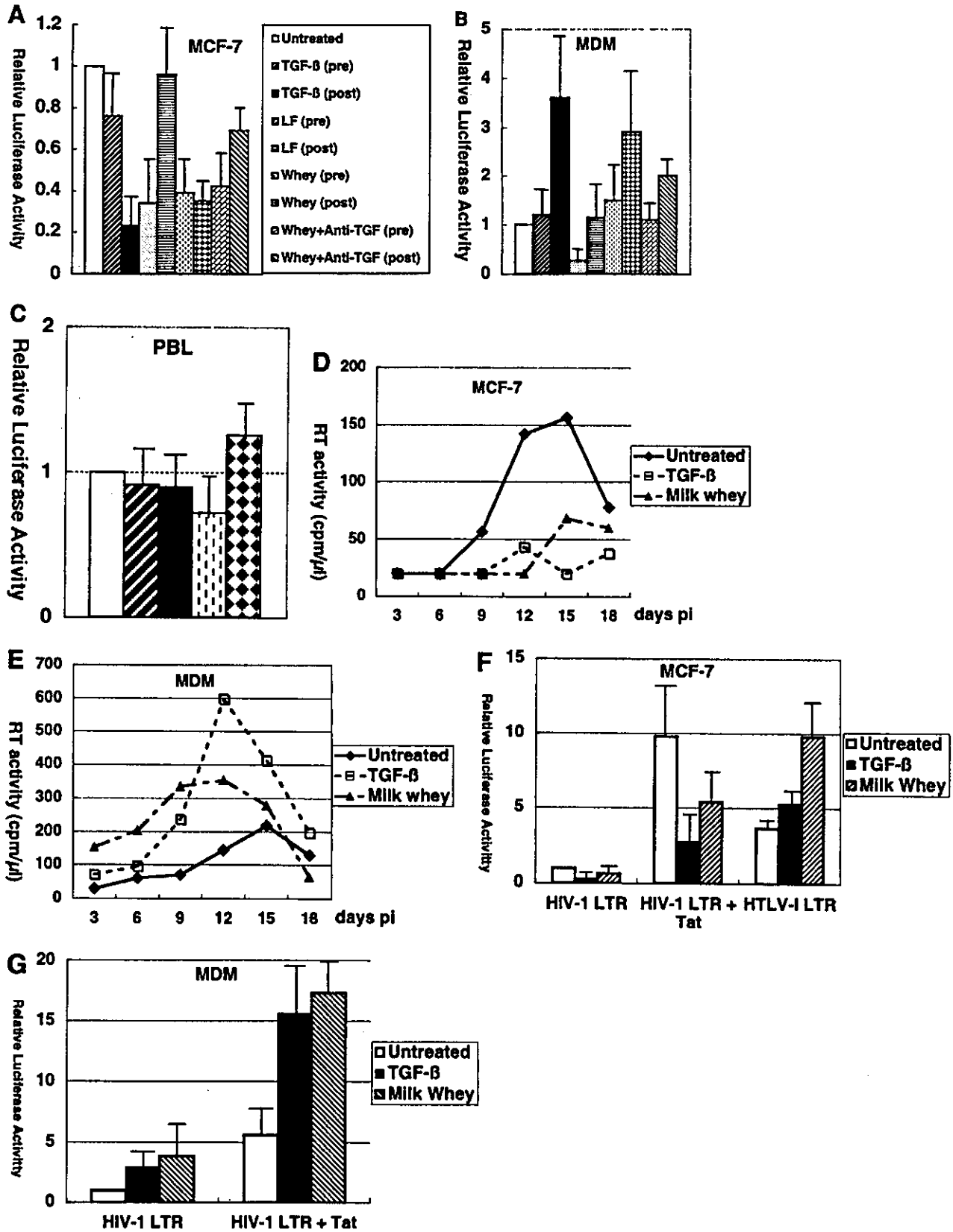

FIG. 2. Cell type-dependent effects of TGF-β on HIV-1 infection. (A, B, and C) In single-round viral replication assays, TGF-β inhibited HIV-1 infection of MCF-7 cells, enhanced HIV-1 infection of monocyte-derived macrophages at a postentry step(s), and had a minimal effect on HIV-1 infection of peripheral blood lymphocytes. MCF-7 cells (A), monocyte-derived macrophages (B), and peripheral blood lymphocytes (C) were either untreated or treated for 16 h before infection (pre) or treated after infection (post) with NL4-3-Luc-R$^-$E$^-$ supplemented with Env

in breast milk. In striking contrast to HIV-1 infection of MCF-7 cells, posttreatment of monocyte-derived macrophages with crude milk whey or TGF-β enhanced HIV-1 infection (Fig. 2B). The effect mediated by posttreatment with crude milk whey was reduced to some extent by neutralizing anti-TGF-β antibody, suggesting that TGF-β contributed to that effect. Pretreatment of monocyte-derived macrophages with either reagent had a minimal effect.

TGF-β as well as crude milk whey had a minimal effect on HIV-1 infection of peripheral blood lymphocytes (Fig. 2C) at the concentration used (200 pg of TGF-β per ml and 10% crude milk whey).

Since crude milk whey or TGF-β appears to have dichotomous effects on HIV-1 infection, depending on cell types and timing of treatment, we tested their effects on multiple rounds of HIV-1 replication. Cells were treated with the reagents for 16 h before infection and throughout the experiments. As shown in Fig. 2D and 2E, TGF-β suppressed HIV-1 replication in MCF-7 cells but enhanced that in monocyte-derived macrophages.

Crude milk whey samples derived from three different donors had similar effects, and results obtained with early and mature milk were comparable (data not shown). Our early and mature milk whey samples contained 1.5 to 2.5 ng and 1.0 to 2.0 ng of TGF-β2 per ml, respectively (data not shown). Since 10% milk whey was used in our experiments, the final concentration of TGF-β would be approximately 200 pg/ml. Therefore, although breast milk must be diluted in baby's gastrointestinal tract, we consider that concentrations of milk whey (10%) and TGF-β (200 pg/ml) were physiologically relevant.

Thus, the net effects of crude milk whey and TGF-β on HIV-1 infection may be negative in MCF-7 cells and positive in monocyte-derived macrophages.

TGF-β downregulates HIV-1 LTR activity in MCF-7 cells but upregulates it in monocyte-derived macrophages. Previous studies have demonstrated that TGF-β upregulates HIV-1 LTR activity in mesangial cells and murine B cell lines. However, since TGF-β plays pleiotropic roles, depending on the cell types involved and combinations with other factors, we tested its effect on HIV-1 LTR activity in MCF-7 cells. As shown in Fig. 2F, HIV-1 LTR activity, either basal and Tat induced, was downregulated by TGF-β in MCF-7 cells. That effect was specific, because TGF-β did not have inhibitory effect on an irrelevant promoter like the HTLV-I LTR at this concentration.

Macrophages are another major cellular components in breast milk and probably serve as an important source of HIV-1 in this body fluid. In similar experiments, TGF-β upregulated basal and Tat-induced HIV-1 LTR activity in monocyte-derived macrophages (Fig. 2G).

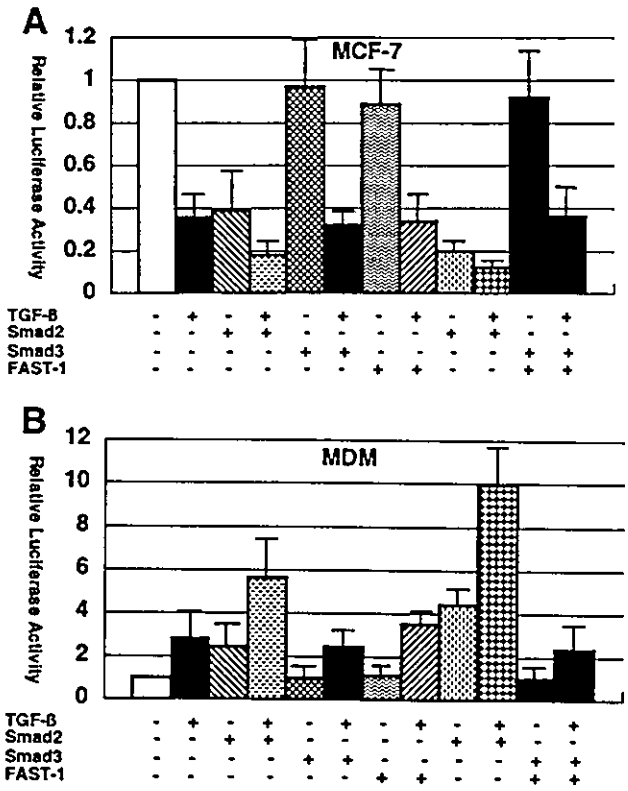

FIG. 3. Augmentation of TGF-β-mediated effects by FAST-1 and Smad2. MCF-7 cells (A) and monocyte-derived macrophages (B) were transfected with pGL-HIV-1-LTR along with pcDNA3.1 or expression vector of the indicated factor and either untreated or treated with TGF-β (200 pg/ml). Results are shown as luciferase activity relative to that of pGL-HIV-1-LTR and pcDNA3.1 when untreated. Error bars represent standard deviations from triplicate experiments.

Overexpression of FAST-1 and Smad2 but not Smad3 augmented TGF-β-mediated effects. TGF-β conveys its signal from membrane receptors into the nucleus by specific mediators called SMADs (reviewed in 11). SMADs activation can lead to transcriptional activation through direct binding to specific DNA sequences and association with another transcription factor such as FAST-1 (32). To further investigate mechanisms whereby TGF-β exerts its effects on HIV-1 LTR, those mediators were overexpressed in transient expression assays.

Cotransfection of Smad2, FAST-1, or both with pGL-HIV-1-LTR augmented TGF-β-mediated effects, downregulation in MCF-7 cells, and upregulation in monocyte-derived macrophages (Fig. 3A and 3B). However, Smad3 alone and in combination with FAST-1 barely influenced HIV-1 LTR activity.

glycoprotein from 89.6 with TGF-β (200 pg/ml), human lactoferrin (LF) (100 ng/ml), or crude milk whey (10%) with and without anti-TGF-β antibody (10 μg/ml). Cells were harvested 48 h after infection, and cell lysates were subjected to luciferase assays. Arbitrary light units are shown. Error bars represent standard deviations from duplicate experiments. (D, E) In multiple-round viral replication assays, TGF-β inhibited HIV-1 infection of MCF-7 cells and enhanced HIV-1 infection of monocyte-derived macrophages. Approximately 2×10^5 MCF-7 cells (D) or 4×10^5 monocyte-derived macrophages (E) were untreated or treated with TGF-β (200 pg/ml) or crude milk whey (10%) for 16 h before and continuously after infection with HIV-1 89.6. The experiments were repeated twice with similar results. Another experiment in which monocyte-derived macrophages were also infected with ELI also gave similar results (data not shown). (F, G) Cell type-dependent effects of TGF-β on HIV-1 LTR activity. MCF-7 cells (F) and monocyte-derived macrophages (G) were transfected with pGL-HIV-1-LTR and pSV2-CAT, pGL-HIV-1-LTR and pSV2-Tat, or pHTLV-I-luc and either untreated or treated with TGF-β (200 pg/ml) or crude milk whey (10%). Results are shown as luciferase activity relative to that of pGL-HIV-1-LTR and pSV2-CAT when untreated. Error bars represent standard deviations from triplicate experiments.

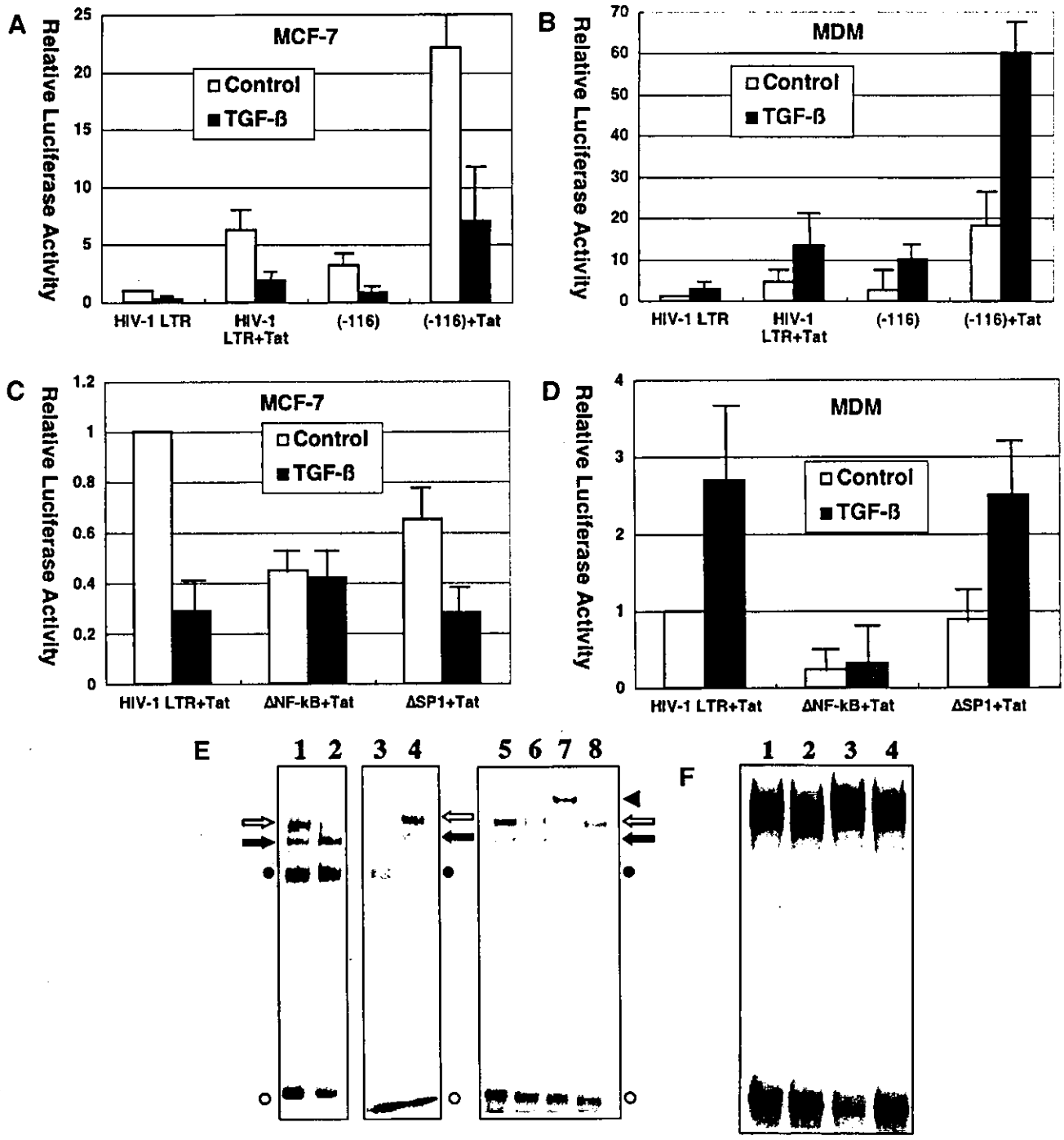

FIG. 4. TGF-β effects on HIV-1 infection are mediated by NF-κB activity. (A to D) Critical role of NF-κB binding sites in TGF-β-mediated effects on HIV-1 LTR. MCF-7 cells (A) or monocyte-derived macrophages (B) were transfected with pGL-HIV-1-LTR or pGL-HIV-1-LTR(−116) along with pSV2-Tat and either untreated or treated with TGF-β (200 pg/ml). Also, MCF-7 cells (C) or monocyte-derived macrophages (D) were transfected with pGL-HIV-1-LTR, pGL-HIV-1-LTRΔNF-κB, or pGL-HIV-1-LTRΔSP1 along with pSV2-Tat and either untreated or treated with TGF-β (200 pg/ml). Error bars represent standard deviations from triplicate experiments. (E, F) TGF-β effects on NF-κB binding to HIV-1 LTR. (E) ^{32}P-labeled oligonucleotides corresponding to sequences spanning the HIV-1 LTR NF-κB binding sites were incubated with nuclear extracts (15 μg of protein) from MCF-7 cells (lanes 1, 2, and 5 to 8) or monocyte-derived macrophages (lanes 3 and 4) and analyzed by gel mobility shift assays. Those cells were either untreated (lanes 1 and 3) or treated (lanes 2 and 4 to 8) with TGF-β (200 pg/ml) for 10 min before harvest. In addition, anti-p65 antibody (lane 6), anti-p50 antibody (lane 7), or control serum (lane 8) was added to the reaction. An open arrow and a solid arrow indicate the p50/p65 heterodimer (NF-κB) and p50 homodimer, respectively. An open circle and a solid circle indicate free probe and nonspecific complex, respectively. A solid triangle indicates a complex supershifted by anti-p50 antibody. (F) ^{32}P-labeled oligonucleotides corresponding to sequences spanning the HIV-1 LTR SP1 binding sites were incubated with nuclear extracts from MCF-7 cells (lanes 1 and 2) or monocyte-derived macrophages (lanes 3 and 4) and analyzed by gel mobility shift assays. Those cells were either untreated (lanes 1 and 3) or treated with TGF-β (200 pg/ml) for 10 min before harvest (lanes 2 and 4).

Thus, Smad2-FAST-1 association apparently plays a role in TGF-β-mediated effects on HIV-1 LTR. It has been shown that hFAST-1 is expressed in all normal human tissues including breast tissue and mediates responses to TGF-β by interacting with Smad2 and Smad4 (32).

Next, we investigated whether Smad2-FAST-1 complex can bind to HIV-1 LTR; however, extensive search with gel mobility shift assay using a panel of oligonucleotides spanning HIV-1 LTR sequence failed to identify a Smad2-FAST-1 binding site(s) (data not shown). Therefore, TGF-β/Smad2/FAST-1 may mediate its effects on HIV-1 LTR indirectly, possibly through transcriptional regulation of other gene(s). Alternatively, TGF-β may mediate SMAD-independent signaling pathway (reviewed in 4).

NF-κB binding sites are critical for TGF-β-mediated effect on HIV-1 LTR. As mentioned above, TGF-β-mediated effects on HIV-1 LTR may be independent of direct binding of SMADs/FAST-1 to HIV-1 LTR and TGF-β signaling may exert its effects on HIV-1 LTR through other *cis*-acting element(s). To demonstrate which *cis*-acting element(s) are involved in TGF-β-mediated effects, a panel of HIV-1 LTR-reporter constructs were tested in transient expression assays. Since a 5′-truncation to −116 relative to transcription start site had little effect on TGF-β-mediated activity (Fig. 4A and 4B), we focused on NF-κB binding sites and SP1 binding sites, major elements located downstream of −116. Deletion of SP1 binding sites had little effect, while deletion of NF-κB binding sites almost abrogated TGF-β-mediated activity in both MCF-7 cells and monocyte-derived macrophages (Fig. 4C and 4D).

To confirm the aforementioned results obtained from transient expression assays, we performed gel mobility shift assays using nuclear extracts from MCF-7 cells that had been untreated and treated with TGF-β. Untreated MCF-7 cells had weak but detectable NF-κB activity. TGF-β treatment of MCF-7 cells abrogated NF-κB activity on HIV-1 LTR, but had little effect on SP1 activity (Fig. 4E and 4F). On the contrary, NF-κB activity in nuclear extracts from untreated monocyte-derived macrophages was enhanced in those from TGF-β-treated monocyte-derived macrophages (Fig. 4E and 4F). It is noteworthy that TGF-β stimulation of HIV-1 LTR through NF-κB activity has been shown in HaCat cells (human keratinocytes) and 300.19 (mouse pre-B) cells (14). These results indicate that TGF-β mediates both inhibitory and stimulatory effects on HIV-1 LTR, at least in part, through the NF-κB pathway.

TGF-β downregulates expression of IκB kinase. In a recent study, TGF-β has been shown to downregulate expression of IκB kinase in mouse mammary epithelial cells. IκB kinase is induced upon various stimuli, degrades IκB, and induces NF-κB activity. To demonstrate whether TGF-β is capable of downregulating IκB kinase expression in MCF-7 cells, real-time RT-PCR was performed. As shown in Fig. 5, TGF-β treatment reduced IκB kinase mRNA levels in MCF-7 cells in a dose-dependent manner but had minimal effect in monocyte-derived macrophages. Thus, IκB kinase appears to be one of the targets of TGF-β action on mammary epithelial cells.

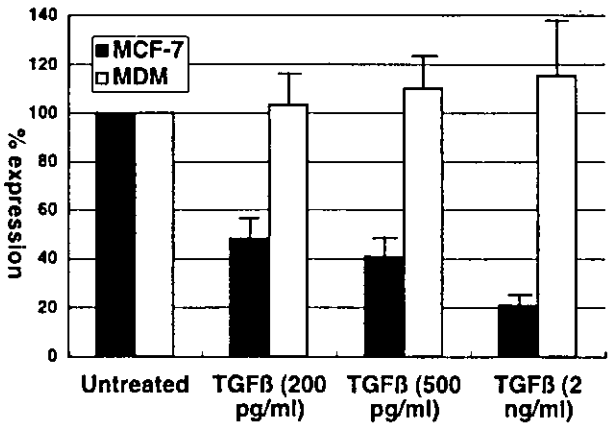

FIG. 5. Regulation of IκB kinase gene expression by TGF-β. MCF-7 cells (solid bars) or monocyte-derived macrophages (open bars) were either untreated or treated with the indicated amount of TGF-β for 60 min, and IκB kinase mRNA levels were determined by real-time RT-PCR. In untreated MCF-7 cells and monocyte-derived macrophages, IκB kinase mRNA levels were approximately 0.1% and 0.3%, respectively, of that of the housekeeping gene tubulin mRNA. The change in expression is shown as expression relative to that in untreated cells. Error bars represent standard deviations from triplicate experiments.

DISCUSSION

Although milk-borne infection accounts for a considerable number of mother-to-child transmissions of HIV-1, the precise molecular and cellular mechanisms by which maternal cell-derived virus is transmitted through the gastrointestinal mucosa to infants remain obscure. In addition to macrophages and lymphocytes, mammary epithelial cells are thought to be the sources of HIV-1 in breast milk. Interestingly, the susceptibility of those cells to HIV-1 strains differs: macrophages support replication of R5-tropic HIV-1 much better than that of X4-tropic HIV-1, lymphocytes support replication of R5- and X4-tropic HIV-1 equally well, and mammary epithelial cells support replication of X4-tropic rather than R5-tropic HIV-1 (18, 28, 30). It is, however, unclear which cell type plays a major role in mother-to-child transmission.

A recent study has shown a differential distribution of HIV-1 variants between breast milk and peripheral blood: the major variant in one compartment corresponded to a minor variant in the other compartment (2). Such observations suggest that a host factor(s) in breast milk can modulate HIV-1 infection. Interestingly, while peripheral blood often harbored both R5- and X4-tropic viruses, HIV-1 variants in breast milk were always R5 tropic (2). Therefore, a breast milk-derived host factor(s) may favor replication of R5-tropic HIV-1 but not X4-tropic HIV-1.

In this study, we identified TGF-β, a major cytokine in breast milk, as a bifunctional modulator of HIV-1 infection. TGF-β favored HIV-1 infection of macrophages but suppressed that of mammary epithelial MCF-7 cells. The pro-HIV-1 effect of TGF-β on macrophages was already reported (13), but it has not been demonstrated how TGF-β influences HIV-1 infection of mammary epithelial cells. TGF-β appears to have dichotomous effects on HIV-1 infection of lymphocytes: it enhanced HIV-1 infection at 10 ng/ml or lower, but suppressed it at 1 ng/ml or higher (26). Peripheral blood lymphocytes were relatively refractory to TGF-β at the concentration (200 pg/ml) used in this study. Since the TGF-β concentration in breast milk is as high as 1 to 2 ng/ml, HIV-1 infection of lymphocytes may be inhibited in this setting.

Thus, the milk-borne cytokine TGF-β may be most beneficial to HIV-1 infection of macrophages, and therefore R5-tropic HIV-1 predominance in breast milk and preferential mother-to-child transmission of R5-tropic HIV-1. However, breast milk contains a number of host factors that variably modulate HIV-1 infection. Lactoferrin inhibits replication of both R5- and X4-tropic HIV-1 in various cell types (24). Prostaglandin E2 inhibits or enhances HIV-1 infection, depending on the virus strains, cell types infected, and the timing of treatment (5, 29). Interestingly, an identified factor in breast milk that is sensitive to cathepsin D has been shown to enhance X4 HIV-1 infection of MCF-7 cells (18). Thus, it appears that the net effect of breast milk on HIV-1 infection depends on the balance of those pro- and anti-HIV-1 factors. Further extensive studies are required for a better understanding of the interaction between HIV-1 and milk-derived host factors.

ACKNOWLEDGMENTS

We thank N. Landau, J. Sodroski, M. Martin, K. Peden, J. L. Wrana, B. Vogelstein, and K. T. Jeang for reagents, S. Chiyoda, H. Okuda, and K. Deguchi (Nagasaki Red Cross Blood Center) for blood samples, and M. Yokoyama for excellent technical assistance and graphic work.

This work was supported in part by a grant provided by a Research for the Future Program (JSPS-RFTF97L00705) of the Japan Society for the Promotion of Science and by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture, Japan.

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JOURNAL OF VIROLOGY, Aug. 2004, p. 8654–8662 Vol. 78, No. 16
0022-538X/04/$08.00+0 DOI: 10.1128/JVI.78.16.8654–8662.2004

Spirodiketopiperazine-Based CCR5 Inhibitor Which Preserves CC-Chemokine/CCR5 Interactions and Exerts Potent Activity against R5 Human Immunodeficiency Virus Type 1 In Vitro

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Received 6 January 2004/Accepted 31 March 2004

We identified a novel spirodiketopiperazine (SDP) derivative, AK602/ONO4128/GW873140, which specifically blocked the binding of macrophage inflammatory protein 1α (MIP-1α) to CCR5 with a high affinity (K_d of ≈3 nM), potently blocked human immunodeficiency virus type 1 (HIV-1) gp120/CCR5 binding and exerted potent activity against a wide spectrum of laboratory and primary R5 HIV-1 isolates, including multidrug-resistant HIV-1 (HIV-1_{MDR}) (50% inhibitory concentration values of 0.1 to 0.6 nM) in vitro. AK602 competitively blocked the binding to CCR5 expressed on Chinese hamster ovary cells of two monoclonal antibodies, 45523, directed against multidomain epitopes of CCR5, and 45531, specific against the C-terminal half of the second extracellular loop (ECL2B) of CCR5. AK602, despite its much greater anti-HIV-1 activity than other previously published CCR5 inhibitors, including TAK-779 and SCH-C, preserved RANTES (regulated on activation normal T-cell expressed and secreted) and MIP-1β binding to CCR5$^+$ cells and their functions, including CC-chemokine-induced chemotaxis and CCR5 internalization, while TAK-779 and SCH-C fully blocked the CC-chemokine/CCR5 interactions. Pharmacokinetic studies revealed favorable oral bioavailability in rodents. These data warrant further development of AK602 as a potential therapeutic for HIV-1 infection.

Highly active antiretroviral therapy has had a major impact on the AIDS epidemic in industrially advanced nations (5, 20); however, eradication of human immunodeficiency virus type 1 (HIV 1) appears to be currently impossible, in part due to the viral reservoirs remaining in blood and infected tissues (6, 27). The limitation of antiviral therapy of AIDS is exacerbated by complicated regimens, the development of drug-resistant HIV-1 variants (11), and a number of inherent adverse effects. Successful antiviral drugs, in theory, exert their virus-specific effects by interacting with viral receptors, virally encoded enzymes, viral structural components, viral genes, or their transcripts without disturbing cellular metabolism or function (20). However, at present, no antiretroviral drugs or agents are likely to be completely specific for HIV-1 or to be devoid of toxicity or side effects in the therapy of AIDS, which has been a critical issue because patients with AIDS and its related diseases will have to receive antiretroviral therapy for a long period of time, perhaps for the rest of their lives (6, 27). Thus, the identification of new antiretroviral drugs which have unique mechanisms of action and produce no or minimal side effects remains an important therapeutic objective (20). In this respect, it has been thought that certain chemokine receptor inhibitors might produce no or minimal toxicity.

In the present study, we designed, synthesized, and identified a novel small nonpeptidic CCR5 inhibitor, AK602/ONO4128/GW873140, and related compounds which showed high binding affinity to CCR5, potently inhibited CCR5 gp120 interactions, and had potent HIV-1-specific antiviral activity against laboratory and clinical strains of HIV-1, including highly drug-resistant HIV-1 variants. We describe here the pharmacological characteristics of AK602/ONO4128/GW873140 and its unique feature that, despite the compound's much greater anti-HIV-1 activity compared to previously published CCR5 inhibitors, AK602/ONO4128/GW873140 preserves RANTES and MIP-1β binding to CCR5$^+$ cells and their functions.

MATERIALS AND METHODS

Reagents. Two newly designed and synthesized spirodiketopiperazine (SDP) derivatives, AK530 [(3*S*)-1-but-2-yn-1-yl-3-[(1*S*)-cyclohexylhydroxymethyl]-9 (3,5-dimethyl-1-phenyl-1H-pyrazol-4-ylmethyl)-1,4,9-triazaspiro[5.5]undecane 2,5-dione dihydrochloride] and AK602 [4-[4-[(3*R*)-1-butyl-3-[(1*R*) cyclohexylhydroxymethyl]-2,5-dioxo-1,4,9-triazaspiro[5.5]undec-9-yl methyl]phenoxy]benzoic acid hydrochloride], are discussed in the present report. The methods for their synthesis and physicochemical profiles will be described elsewhere. The structures of these two compounds are shown in Fig. 1. A previously reported prototypic SDP derivative, E913 (17), was used as a reference compound. E921 and

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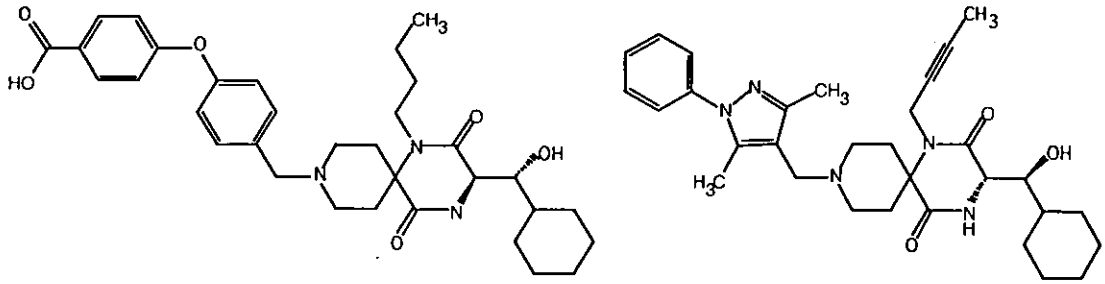

AK602 Mr. 614.2 AK530 Mr. 604.6

FIG. 1. Structures of AK602 and AK530.

AK671, which have the same structures as CCR5 inhibitors TAK-779 and SCH-351125 (SCH-C), respectively, were synthesized as previously described by others (1, 28).

Zidovudine was purchased from Sigma (St. Louis, Mo.). Nelfinavir and saquinavir were provided by Japan Energy (Tokyo, Japan) and Roche Products (Welwyn Garden City, United Kingdom), respectively.

^{125}I-labeled chemokines macrophage inflammatory protein-1α (MIP-1α), macrophage inflammatory protein-1β (MIP-1β), and RANTES were purchased from Amersham Pharmacia Biotech (Little Chalfont, United Kingdom) and PerkinElmer Life Sciences, Inc. (Boston, Mass.), and three corresponding unlabeled chemokines (MIP-1α, MIP-1β, and RANTES) were purchased from PeproTech Inc. (Rocky Hill, N.J.). Recombinant HIV-1_{YU2} gp120 (rgp120) and human soluble CD4 (sCD4) were purchased from Immuno Diagnostics, Inc. (Woburn, Mass.).

Cells, viruses, and anti-HIV-1 assay. Chinese hamster ovary (CHO) cells expressing CCR5 (17) were maintained in Ham's F-12 medium (Gibco-BRL, Rockville, Md.) supplemented with 10% fetal calf serum (JRH Biosciences, Lenaxa, Kans.), 50 U of penicillin per ml, and 50 μg of streptomycin per ml in the presence of 5 μg of blasticidin S hydrochloride per ml. Peripheral blood mononuclear cells were isolated from buffy coats of HIV-1-seronegative individuals with Ficoll-Hypaque density gradient centrifugation and cultured at a concentration of 10^6 cells/ml in RPMI 1640-based culture medium supplemented with 10% fetal calf serum and antibiotics with 10 μg of phytohemagglutinin per ml for 3 days prior to use (phytohemagglutinin-peripheral blood mononuclear cells). Cell line CCR5$^+$ MOLT4 (18) was a kind gift from Yosuke Maeda, Kumamoto University, Japan.

A panel of HIV-1 strains was employed for drug susceptibility assays: HIV $1_{Ba\text{-}L}$ (8), HIV-$1_{JR\text{-}FL}$ (13) HIV-$1_{NL4\text{-}3}$ (34), a wild-type HIV-1_{MOKW} isolated from a drug-naive AIDS patient (17), and two multidrug-resistant (HIV-1_{MDR}) primary HIV-1 strains (HIV-1_{JSL} and HIV-1_{MM}) (36). All primary HIV-1 strains were passaged once or twice in phytohemagglutinin-peripheral blood mononuclear cell cultures, and the culture supernatants were stored at −80°C until use. Antiviral assays with phytohemagglutinin-peripheral blood mononuclear cells were conducted as previously reported (12, 17, 26).

HIV-1 gp120 binding inhibition assays. CCR5$^+$ CHO cells were incubated with rgp120 (5 μg/ml) and sCD4 at 5 μg/ml, biotinylated with EZ-link sulfo-NHS-SS-biotin (Pierce, Rockford, Ill.) in the presence of the indicated concentrations of a CCR5 inhibitor for 1 h at 37°C. Cells were washed, and the binding of the rgp120-sCD4 complex to CCR5$^+$ CHO cells was determined with phycoerythrin-conjugated streptavidin (BD PharMingen, San Diego, Calif.). Nonspecific binding was determined based on the mean florescence intensity of phycoerythrin-conjugated streptavidin with sCD4 but without rgp120. Drug concentrations that brought about 50% inhibition (IC_{50}) of mean fluorescence intensity were then determined.

Generation of ^{3}H-labeled CCR5 inhibitors. Five CCR5 inhibitors, AK530, AK602, E913, E921/TAK-779, and AK671/SCH-C, were tritiated by reductive amination with sodium triacetoxyborotritide (10), methylation with [^{3}H]methyliodide, and heterogeneous catalytic exchange with tritium gas (4). Detailed description of the radiosynthesis of the inhibitors will be presented by J.M. elsewhere. In brief, [^{3}H]E913, [^{3}H]AK530, and [^{3}H]AK602 were prepared by reductive amination of the corresponding aldehyde with piperidine-containing components of each inhibitor with an excess of sodium triacetoxyborotritide, and the tritium label was positioned selectively into the methylene group connecting the two components, generating inhibitors with specific activities of 10.2 Ci/mmol, 17.5 Ci/mmol, and 8.3 Ci/mmol, respectively. [^{3}H]E921/TAK-779 was prepared by methylating the *N*-methyl precursor of E921/TAK-779 with [^{3}H]methyliodide, generating [^{3}H]E921/TAK-779, with a specific activity of 6.1 Ci/mmol. For the preparation of [^{3}H]AK671/SCH-C, methyl-2,4-dimethylpyridine-3-carboxylate was tritiated by an exchange with tritium gas, catalyzed by palladium on carbon in ethanol and triethylamine. Its conversion to N-oxide and alkaline hydrolysis of the resulting ester provided [^{3}H]2,4-dimethyl-pyridine-3-carboxylic acid. Its condensation with *N-tert*-butoxycarbonyl precursor provided [^{3}H]AK671/SCH-C, with a specific activity of 5 Ci/mmol.

Saturation binding assay. CCR5$^+$ CHO cells (1.5×10^5 cells/well) were plated onto 48-well, flat-bottomed culture plates, incubated for 24 h, rinsed with Ham's F-12 medium containing 20 mM HEPES and 0.5% bovine serum albumin (Sigma), exposed to various concentrations of each ^{3}H-labeled CCR5 inhibitor, washed thoroughly with cold phosphate-buffered saline, and lysed with 0.5 ml of 1 N NaOH, and the radioactivity in the lysates was measured. The nonspecific binding of a radiolabeled compound was determined based on the radioactivity detected in the CCR5$^+$ CHO cell-plated wells containing the same amount of the ^{3}H-labled CCR5 inhibitor and a 200-fold greater amount of the corresponding non radiolabeled compound. The K_d (dissociation) values of CCR5 inhibitors and the maximal binding values (B_{max} = number of CCR5/cell) were calculated based on their specific radioactivity with Graphpad Prism software (Intuitive Software for Science, San Diego, Calif.). All assays were performed in duplicate, and the values shown in this report are the arithmetic means (±1 standard deviation) of 3 to 10 independently conducted assays.

Chemokine binding inhibition and chemotaxis inhibition assays. CCR5$^+$ CHO cells (1.5×10^5) were plated onto 48-well microculture plates, incubated for 24 h, rinsed, exposed to 3 nM [^{125}I]MIP-1α, [^{125}I]MIP-1β, or [^{125}I]RANTES in the presence of various concentrations of a CCR5 inhibitor at room temperature for 1 h, thoroughly washed with phosphate-buffered saline, and lysed with 0.5 ml of 1 N NaOH, and their radioactivity was counted. The nonspecific binding of the labeled chemokine to the cells was determined based on the radioactivity detected in the wells plated with the same number of CCR5-negative CHO (CHO-K1) cells exposed to each radiolabeled chemokine (3 nM).

Chemotaxis inhibition assays were conducted with CCR5$^+$ MOLT4 cells and the ChemTx System (Neuro Probe, Inc., Gaithersburg, Md.). In brief, CCR5$^+$ MOLT4 cells were exposed to various concentrations of each CCR5 inhibitor for 30 min, thoroughly rinsed, plated onto the upper chamber of the ChemTx System, exposed to 0.5 nM RANTES contained in the lower chamber, and incubated for 4 h at 37°C, and the number of the cells which migrated from the upper chamber to the lower chamber was determined. Percent chemotaxis was determined with the formula 100 × [(number of CCR5 inhibitor-exposed cells which migrated to the lower chamber in the presence of RANTES) − (number of CCR5 inhibitor-unexposed cells which migrated to the lower chamber in the absence of RANTES)]/[(number of CCR5 inhibitor-unexposed cells which migrated to the lower chamber in the presence of RANTES) − (number of CCR5 inhibitor-unexposed cells which migrated to the lower chamber in the absence of RANTES)].

FACS analysis. Fluorescence-activated cell sorting (FACS) analysis was performed as previously described (17) with minor modifications. Briefly, CCR5$^+$ CHO cells (3×10^5) were stained with a phycoerythrin- or fluorescein isothiocyanate-conjugated anti-CCR5 monoclonal antibody 2D7 (BD PharMingen, San Diego, Calif.) or 45523 or 45531 (R&D Systems, Minneapolis, Minn.), with or without a test CCR5 inhibitor, washed, and examined with an Epics XL (Beckman Coulter, Fullerton, Calif.).

RESULTS

Potent activity of AK602 against R5 wild-type and multidrug-resistant R5 HIV-1. We have previously reported that a prototypic SDP derivative, E913, was active against R5 HIV-1 in vitro, with IC_{50} values of 30 to 60 nM as tested in target phytohemagglutinin-treated peripheral blood mononuclear cells (17). Following optimization for increased potency against R5 HIV-1 and favorable pharmacokinetic features, we identified AK602 as the most potent agent among newly designed and synthesized SDP derivatives. AK602 exerted potent activity against three wild-type R5 HIV-1 strains (HIV-$1_{Ba\text{-}L}$, HIV $1_{JR\text{-}FL}$ and HIV-1_{MOKW}) with IC_{50} values of 0.1 to 0.4 nM (Table 1). It was of note that AK602 was substantially more potent than two previously published CCR5 inhibitors, E921/TAK-779 and AK671/SCH-C (1, 28).

During the extended study of the antiviral activity of the prototypic E913, we noted that its activity against R5 HIV-$1_{Ba\text{-}L}$ in vitro varied substantially; the range of IC_{50} values spanned from 14 to 650 nM (Fig. 2). When we tested the activity of E921/TAK-779 in phytohemagglutinin-treated peripheral blood mononuclear cells from multiple seronegative donors, its variability was also substantial: its IC_{50} values varied from 2 to 200 nM. However, when we tested AK602, the variability of AK602's anti-HIV-1 activity was limited and similar to that seen for zidovudine. The difference in the range of the CCR5 inhibitor's IC_{50} values seems to correlate with the potency of the inhibitor examined. Indeed, we have seen a greater variability in the antiviral activity of the prototypic E913 (Fig. 2). Moreover, AK602 suppressed the infectivity and replication of two HIV-1_{MDR} variants, HIV-1_{MM} and HIV-1_{JSL} (36), at extremely low concentrations (IC_{50} values of 0.4 to 0.6 nM), while these two R5 HIV-1 variants were less susceptible to zidovudine, nelfinavir, and saquinavir (IC_{50} values were greater by factors of 10 to 36, >83, and 27 to 32, respectively, compared to those against HIV-$1_{Ba\text{-}L}$). As expected, none of these CCR5 inhibitors suppressed the infectivity and replication of X4 HIV-$1_{NL4\text{-}3}$ in vitro. Although certain CC-chemokines reportedly enhance the replication of X4 HIV-1 (19, 22), no such enhancement of X4 HIV-1 replication was seen with the CCR5 inhibitors examined in this study at concentrations of up to 1 μM (data not shown).

CCR5 binding properties of SDP derivatives. We determined the CCR5 binding profiles of SDP derivatives and compared them with those of previously published CCR5 inhibitors in saturation binding assays employing ^3H-labeled compounds. Figure 3A depicts the CCR5 binding profile of AK602, showing that it binds with high affinity to CCR5. The K_d values thus determined for AK602, E913, E921/TAK-779, and AK671/SCH-C were 2.9 ± 1.0 (Fig. 3A), 111.7 ± 3.5, 32.2 ± 9.6, and 16.0 ± 1.5 nM (data not shown), respectively.

We also asked whether the SDP derivatives blocked the binding to CCR5 of rgp120 following exposure to sCD4. As shown in Fig. 3B, AK602 potently blocked rgp120/sCD4 binding to CCR5 with an IC_{50} value of 2.7 nM, followed by E921/TAK-779 and AK-671/SCH-C, with IC_{50} values of 12.0 and 16.5 nM, respectively. When we asked whether AK602 blocked the intracellular Ca^{2+} mobilization induced by MIP-1α, MDC, SDF-1α, and MCP-1, whose primary receptors are CCR5, CCR4, CXCR4, and CCR2, respectively, with the method we

TABLE 1. Anti-HIV-1 activity of SDP derivatives

Compound	Mean IC_{50} (IC_{90}) ± SD in p24 assay (nM)						CC_{50}[a] (μM)
	HIV-$1_{Ba\text{-}L}$ (R5)	HIV-$1_{JR\text{-}FL}$ (R5)	HIV-1_{MOKW}[b] (R5)	HIV-1_{MM}[b] ($R5_{MDR}$)	HIV-1_{JSL}[b] ($R5_{MDR}$)	HIV-$1_{NL4\text{-}3}$ (X4)	
AK602	0.4 ± 0.3 (12 ± 10)	0.1 ± 0.1 (4 ± 2)	0.2 ± 0.1 (5 ± 3)	0.6 ± 0.2 (11 ± 2)	0.4 ± 0.3 (7 ± 2)	>1,000	50
AK530	32 ± 27 (324 ± 120)	13 ± 4 (144 ± 60)	ND[c]	ND	ND	>1,000	60
E913	82 ± 58 (709 ± 256)	81 ± 46 (>1,000)	51 ± 14 (941 ± 201)	61 ± 28 (>1,000)	64 ± 30 (713 ± 405)	>1,000	50
E921/TAK-779	28 ± 32 (256 ± 169)	5 ± 1 (237 ± 25)	11 ± 7 (194 ± 168)	14 ± 8 (352 ± 180)	7 ± 4 (316 ± 151)	>1,000	50
AK671/SCH-C	4 ± 2 (79 ± 52)	2 ± 0.5 (56 ± 57)	2 ± 1 (54 ± 20)	3 ± 0.5 (138 ± 25)	2 ± 0.3 (84 ± 18)	>1,000	>100
Zidovudine	7 ± 4 (48 ± 21)	10 ± 9 (157 ± 72)	6 ± 5 (47 ± 20)	250 ± 98 (>1,000)	70 ± 64 (>1,000)	11 ± 5 (181 ± 90)	>100
Nelfinavir	12 ± 8 (105 ± 48)	ND	14 ± 8 (82 ± 56)	>1,000	>1,000	20 ± 7 (75 ± 52)	ND
Saquinavir	11 ± 5 (60 ± 21)	ND	5 ± 2 (49 ± 40)	300 ± 65 (>1,000)	350 ± 105 (>1,000)	10 ± 4 (48 ± 2)	ND

[a] Cytotoxic concentrations of a compound that reduces the number of cells by 50% (CC_{50}) were determined as previously reported (17).
[b] HIV-1_{MOKW} was isolated from a drug-naive AIDS patient (17), while HIV-1_{MM} and HIV-1_{JSL} were from patients who received antiretroviral therapy for a long period and whose virus acquired a number of mutations in the RT- and PR-encoding HIV-1 genes (36).
[c] ND, not determined.

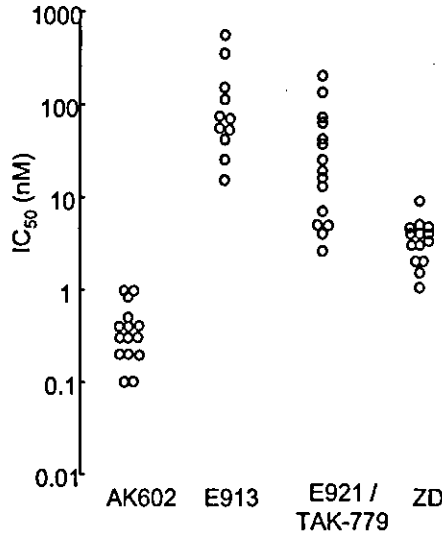

FIG. 2. Variability of anti-HIV-1 activity of AK602 in phytohemagglutinin-peripheral blood mononuclear cells. The range of IC_{50} values of E913 and E921/TAK-779 against HIV-1_{Ba-L} varied substantially when examined in multiple phytohemagglutinin-peripheral blood mononuclear cells as target cells, 14 to 650 nM (n = 11) and 2 to 200 nM (n = 15), respectively, while that of AK602 was relatively narrow, 0.1 to 1 nM (n = 15), similar to that of zidovudine (ZDV), 1 to 9 nM (n = 14).

published previously (17), AK602 completely blocked MIP-1α-induced Ca^{2+} mobilization at 0.1 μM and beyond; however, it failed to block Ca^{2+} mobilization induced with MDC, SDF-1α, and MCP-1 (data not shown).

We also attempted to illustrate where AK602 binds on the CCR5 molecule by employing several monoclonal antibodies known to bind to different domains of CCR5. FACS analyses revealed that there was no AK602 inhibition of the binding of monoclonal antibody 2D7, known to bind to the N-terminal half (or domain A) of the second extracellular loop of CCR5 (14) (Fig. 4). In contrast, AK602 competitively blocked the binding of two different monoclonal antibodies, 45523, reportedly directed against multidomain epitopes of CCR5, and 45531, which is known to be specific against the C-terminal half (or domain B) of the second extracellular loop (ECL2B) of CCR5 (14), as examined with $CCR5^+$ CHO cells (Fig. 4). These data suggest that the potent activity of AK602 against R5 HIV-1 stems from its binding to ECL2B and/or its vicinity with high affinity, resulting in inhibition of gp120/CD4 binding to CCR5. It was of note, however, that another SDP derivative, AK530, whose antiviral activity was moderate (the IC_{50} value against HIV-1_{Ba-L} was 32 nM; Table 1), whose rgp120/sCD4 binding inhibition was the lowest among the inhibitors examined (IC_{50}, 280 nM; Fig. 3B), and had only a moderate effect on the binding of monoclonal antibody 45531 to $CCR5^+$ cells (data not shown), had the highest binding affinity to CCR5 (K_d value, 0.4 nM; data not shown) among the SDP derivatives, suggesting that the binding pocket (or subsite) of certain SDP derivatives (such as AK530) does not quite overlap that of AK602.

SDP derivatives bind to CCR5 but permit RANTES and MIP-1β to bind to CCR5. We asked whether SDP derivatives blocked the binding of CC-chemokines to CCR5 expressed on the surface of CHO cells with [^{125}I]RANTES, [^{125}I]MIP-1α, and [^{125}I]MIP-1β and CCR5 inhibitors AK602, AK530, E921/TAK-779, and AK671/SCH-C. As shown in Fig. 5A, the concentrations of E921/TAK-779 and AK671/SCH-C which

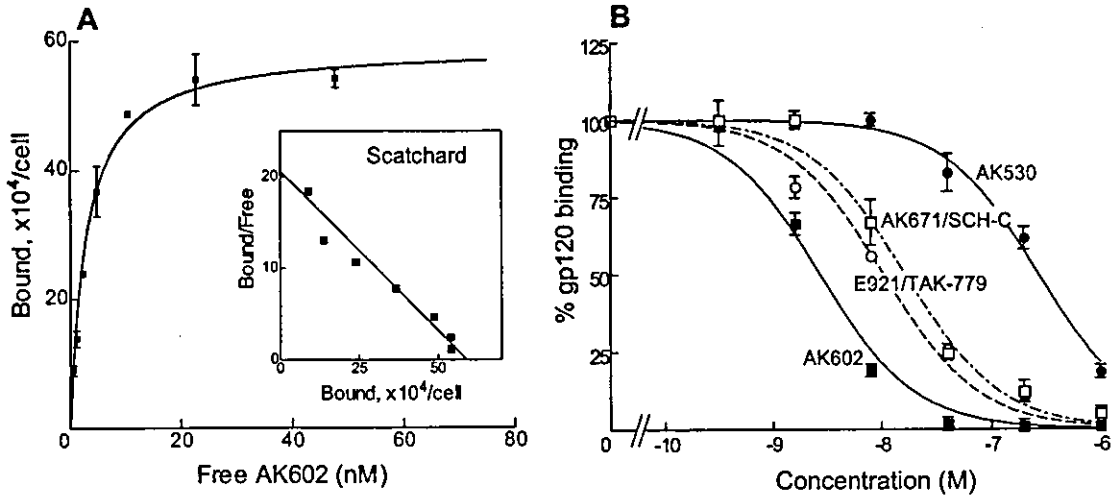

FIG. 3. CCR5 binding profiles and rgp120 binding blocking of various CCR5 inhibitors. (A) Binding affinity of AK602 to CCR5. $CCR5^+$ CHO cells were incubated with the ^{3}H-labeled CCR5 inhibitors AK530, AK602, E913, E921/TAK-779, and AK671/SCH-C for 1 h. Following thorough washing, cells were lysed, the radioactivity in the lysates was determined, and B_{max} and K_d values were calculated. The K_d values thus obtained were 0.4 ± 0.4, 2.9 ± 1.0, 111.7 ± 3.5, 32.2 ± 9.6, and 16.0 ± 1.5 nM, respectively. All assays were independently performed 3 to 10 times, and the values represent the arithmetic means ± 1 standard deviation. (B) AK602 potently blocks the binding of rgp120/sCD4 to CCR5. $CCR5^+$ CHO cells were incubated with rgp120 (5 μg/ml) and sCD4 (5 μg/ml) in the presence or absence of the indicated concentrations of CCR5 inhibitors, and the binding of rgp120/sCD4 complex to $CCR5^+$ CHO cells was determined. The 50% binding inhibition (EC_{50}) value was determined based on the mean fluorescence intensity values obtained with or without CCR5 inhibitors. EC_{50} values for AK602, AK530, E921/TAK-779, and AK671/SCH-C were 2.7, 280, 12.0, and 16.5 nM, respectively.

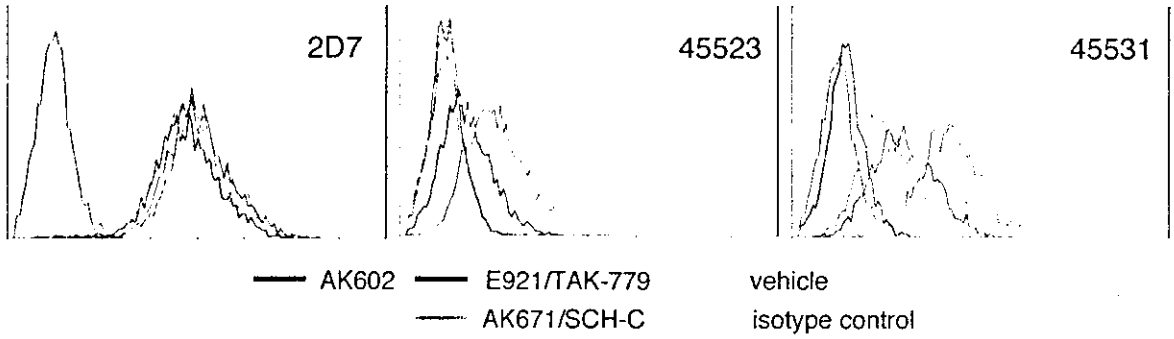

FIG. 4. AK602 binds to the second extracellular loop of CCR5. AK602 at 100 nM almost completely inhibited the binding of two monoclonal antibodies, 45523, directed against multidomain epitopes of CCR5, and 45531, recognizing ECL2B of CCR5. In contrast, E921/TAK-779 and AK671/SCH-C moderately blocked the binding of 45523 and 45531. Note that there was no AK602 inhibition of the binding of a monoclonal antibody 2D7, which is known to bind to domain A of ECL2 of CCR5.

blocked RANTES binding to CCR5 by 50% (IC_{50}) were 110 and 40 nM, respectively, and RANTES binding was completely blocked in the presence of ≥10 μM E921/TAK-779 or AK671/SCH-C. In contrast, AK602 only partially blocked RANTES binding to CCR5 by 40% even at 10 μM (Fig. 5A). The binding of MIP-1β to CCR5 was also completely blocked by E921/TAK-779 and AK671/SCH-C; however, AK602 failed to completely block MIP-1β binding (Fig. 5B). The MIP-1β binding value in the presence of 10 μM AK602 was 10%, and no further blockade occurred at higher concentrations up to 40 μM (data not shown). AK530 also failed to completely block the binding of RANTES and MIP-1β to CCR5.

These data suggest that the binding pockets (or subsites) of CCR5 for SDP derivatives only partially overlap the CC-chemokine binding sites of CCR5 or that the conformational changes ensuing the binding of SDP derivatives to CCR5 have only moderate effects on the binding of RANTES and MIP-1β. In the initial search for CCR5 inhibitors, lead compounds were sought as those inhibiting MIP-1α binding to CCR5 and MIP-1α-driven cytosolic Ca^{2+} flux, and thus, as expected, AK602 blocked MIP-1α binding to CCR5 although AK530 was substantially less potent in blocking MIP-1α binding (Fig. 5C). E921/TAK-779 and AK671/SCH-C were also found to completely block MIP-1α binding to CCR5 (Fig. 5C).

AK602 and RANTES bind simultaneously to CCR5. As described above, AK602 and AK530 only partially inhibited RANTES binding to $CCR5^+$ CHO cells; however, it was not clear whether those SDP derivatives and RANTES bound simultaneously to CCR5. Therefore, competitive binding assays employing ^3H-labeled and unlabeled AK602 and ^{125}I-labeled and unlabeled RANTES were conducted. As shown in Fig. 6A, the binding of [^3H]AK602 (10 nM) to CCR5 was only partially inhibited by ≥4 nM RANTES. Also, the binding of [^{125}I]RANTES at 8 nM was only inhibited by up to 20% in the presence of 10 nM AK602 (Fig. 6B).

The interpretation that AK602 and RANTES bind simultaneously to CCR5 was corroborated by another experiment in which a lower concentration of [^3H]AK602 and much higher concentrations of RANTES were used (Fig. 6A, inset). The radioactivity counted for [^3H]AK602 (5 nM) bound to $CCR5^+$ CHO cells was only moderately blocked in the presence of 100 and 1,000 nM RANTES, by 32 and 46%, respectively (Fig. 6A, inset). These data suggest that the SDP derivatives, in particular AK602, and RANTES bind simultaneously to CCR5, al-

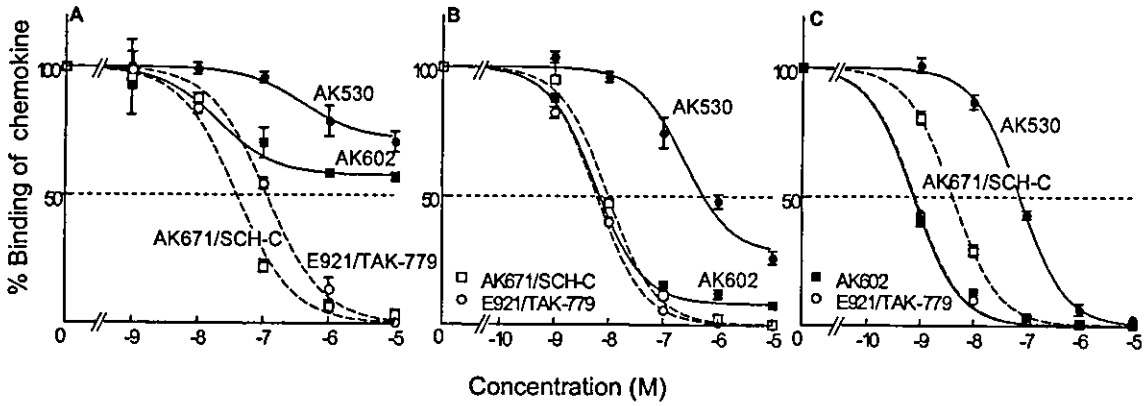

FIG. 5. Inhibition of CC-chemokine binding to CCR5 by various CCR5 inhibitors. $CCR5^+$ CHO cells were incubated with 3 nM [^{125}I]RANTES (A), [^{125}I]MIP 1 β (B), or [^{125}I]MIP-1 α (Pncl C) in the presence and absence of various concentrations of CCR5 inhibitors. Note that while AK671/SCH-C and E921/TAK-779 completely inhibited the binding of [^{125}I]RANTES, [^{125}I]MIP-1α, and [^{125}I]MIP-1β to CCR5, SDP derivatives partially blocked RANTES (A) and MIP-1β (B) binding, although they completely blocked MIP-1α binding (C).

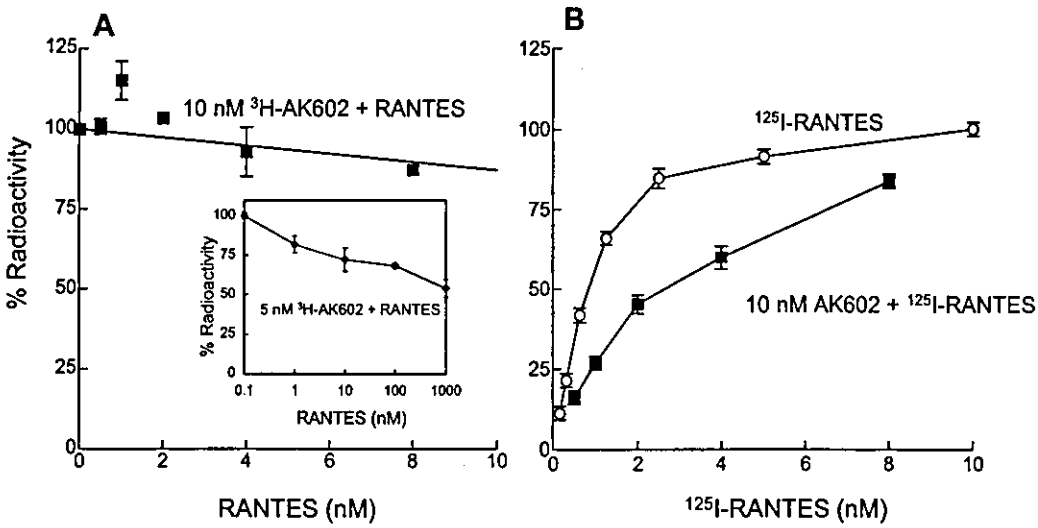

FIG. 6. AK602 and RANTES bind simultaneously to CCR5. (A) CCR5$^+$ CHO cells were exposed to 10 nM [^{3}H]AK602 and various concentrations of unlabeled RANTES. After 1 h of incubation, the cells were washed, and the [^{3}H]AK602 bound to the cells was measured. Note that 100% radioactivity on the ordinate denotes the radioactivity of cell-bound [^{3}H]AK602 without RANTES and that ≈90% of CCR5 molecules are bound to AK602 at 10 nM (Fig. 3A). (B) CCR5$^+$ CHO cells were exposed to 10 nM unlabeled AK602 and various concentrations of [^{125}I]RANTES. After 1 h of incubation, the cells were washed, and the [^{125}I]RANTES bound to the cells was measured. The binding profile of [^{125}I]RANTES alone is illustrated by open circles. Note that 100% radioactivity is equated to the radioactivity of cell-bound [^{125}I]RANTES at 10 nM. The K_d values of RANTES in the presence and absence of 10 nM AK602 were 4.5 and 0.6 nM, respectively.

though conformational changes potentially caused by either of the two might have occurred. Indeed, 15 to 25% inhibition was seen at nearly equimolar concentrations of AK602 and RANTES, which may reflect the involvement of the conformational changes caused by either of the two agents or an overlap in their binding sites (or domains).

AK602 permits RANTES-induced chemotaxis and CCR5 internalization at anti-HIV-1 activity-exerting concentrations. We next asked whether AK602 allowed RANTES-induced chemotaxis and CCR5 internalization with CCR5$^+$ MOLT4 cells and CCR5$^+$ CHO cells at its anti-HIV-1 activity-exerting concentrations. As shown in Fig. 7A, AK671/SCH-C most potently blocked chemotaxis, followed by E921/TAK-779. The chemotaxis values at the IC_{50}s against R5 HIV-1_{Ba-L} of AK671/SCH-C and E921/TAK-779 (4 and 24 nM, respectively: Table 1) were low, 18 and 8%, respectively, suggesting that these two inhibitors considerably blocked chemotaxis at their anti-HIV-1 IC_{50} concentrations as determined in peripheral blood mononuclear cells. In contrast, the chemotaxis seen at the IC_{50} level of AK602, 0.4 nM (see Table 1), was considerable, with 70% retained (Fig. 7A), while that seen AK530 was much less (30%).

In order to corroborate the modest chemotaxis inhibition seen with AK602, the inhibition of RANTES-induced CCR5 internalization was also examined. In the absence of CCR5 inhibitors, ≈50% of CCR5 molecules were internalized from the surface of CCR5$^+$ CHO cells incubated for 1 h at 37°C in the presence of 10 nM RANTES; however, AK671/SCH-C and E921/TAK-779 at 100 nM considerably blocked internalization, and only 19 and 6%, respectively, of CCR5 molecules were internalized. In the presence of higher concentrations of AK671/SCH-C and E921/TAK-779, 300 and 1,000 nM, virtually no CCR5 internalization occurred (Fig. 7B). In contrast, AK530 and AK602 at 100 nM allowed RANTES-induced CCR5 internalization of 46 and 30%, respectively, and even at 300 and 1,000 nM, 10 to 34% CCR5 internalization occurred (Fig. 7B).

DISCUSSION

A novel SDP derivative, AK602/ONO4128/GW873140, exhibited high affinity to CCR5, blocked rgp120/sCD4 complex binding to CCR5, and exerted potent activity against a wide spectrum of laboratory and primary R5 HIV-1 isolates, including HIV-1_{MDR}. We recently examined AK602 against several non-clade B R5 HIV strains and found that in general AK602 is comparably active against such non-clade B strains (data not shown). It is of note that several small-molecule CCR5 inhibitors have been reported in the literature, including SCH-D (D. Schurmann et al., Abstr. 11th Conf. Retroviruses Opportunistic Infections, 2004, abstr. 140LB), UK427,857 (A. L. Pozniak et al. Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., 2003, abstr. H-443), CMPD167 (32), and TAK-220 (Y. Iizawa et al., Abstr. 10th Conf. Retroviruses Opportunistic Infections, 2003, abstr. 11).

In the present study, we also demonstrated that AK602 potently blocked rgp120/sCD4 complex binding to CCR5. With respect to gp120/CD4 binding to CCR5, Olson et al. previously reported no correlation between fusion with and entry into the target cell of HIV-1 and inhibition of rgp120/sCD4 complex binding to CCR5, based on data with various anti-CCR5 monoclonal antibodies (24). However, with all small-molecule SDP derivatives examined in the present study, inhibition of HIV-1 infectivity and replication generally correlated with inhibition of the rgp120/sCD4 complex binding to CCR5, strongly suggesting that the anti-HIV-1 activity of SDP