V region mutation and selection in germinal center B cells. *J. Exp. Med. 178:1293.*
17. Cumano, A., and K. Rajewsky. 1986. Clonal recruitment and somatic mutation in the generation of immunological memory to the hapten NP. *EMBO J. 5:2459.*
18. Allen, D., T. Simon, F. Sablitzky, K. Rajewsky, and A. Cumano. 1988. Antibody engineering for the analysis of affinity maturation of an anti-hapten response. *EMBO J. 7:1995.*
19. French, D. L., R. Laskov, and M. D. Scharff. 1998. The role of somatic hypermutation in the generation of antibody diversity. *Science 244:1152.*
20. Poignard, P., T. Fouts, D. Naniche, J. P. Moore, and Q. J. Sattentau. 1996. Neutralizing antibodies to human immunodeficiency virus type-1 gp120 induce envelope glycoprotein subunit dissociation. *J. Exp. Med. 183:473.*
21. Kimpton, J., and M. Emerman. 1992. Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated β-galactosidase gene. *J. Virol. 66:2232.*
22. Matsushita, S., H. Maeda, K. Kimachi, Y. Eda, Y. Maeda, T. Murakami, S. Tokiyoshi, and K. Takatsuki. 1992. Characterization of a mouse/human chimeric monoclonal antibody (Cβ1) to a principal neutralizing domain of the human immunodeficiency virus type 1 envelope protein. *AIDS Res. Hum. Retroviruses 8:1107.*
23. Wu, X., J. Feng, A. Komori, E. C. Kim, H. Zan, and P. Casali. 2003. Immunoglobulin somatic hypermutation: double-strand DNA breaks, AID and error-prone DNA repair. *J. Clin. Immunol. 23:235.*
24. Honjo, T., K. Kinoshita, and M. Muramatsu. 2002. Molecular mechanism of class switch recombination: linkage with somatic hypermutation. *Annu. Rev. Immunol. 20:165.*
25. Storb, U., and J. Stavnezer. 2002. Immunoglobulin genes: generating diversity with AID and UNG. *Curr. Biol. 12:R725.*
26. Jacobs, H., and L. Bross. 2001. Towards an understanding of somatic hypermutation. *Curr. Opin. Immunol. 13:208.*
27. van Eijk, M., T. Defrance, A. Hennino, and C. de Groot. 2001. Death-receptor contribution to the germinal-center reaction. *Trends Immunol. 22:677.*
28. Bailis, J. M., and S. L. Forsburg. 2004. MCM proteins: DNA damage, mutagenesis and repair. *Curr. Opin. Genet. Dev. 14:17.*
29. Khuda, S. E., M. Yoshida, Y. Xing, T. Shimasaki, M. Takeya, K. Kuwahara, and N. Sakaguchi. 2004. The *Sac3* homologue *shd1* is involved in mitotic progression in mammalian cells. *J. Biol. Chem. 279:46182.*
30. Fischer, T., S. Rodriguez-Navarro, G. Pereira, A. Racz, E. Schiebel, and E. Hurt. 2004. Yeast centrin Cdc31 is linked to the nuclear mRNA export machinery. *Nat. Cell Biol. 6:840.*
31. Bauer, A., and R. Kölling. 1996. Characterization of the SAC3 gene of *Saccharomyces cerevisiae. Yeast 12:965.*
32. Mirnics, Z. K., E. Caudell, Y. Gao, K. Kuwahara, N. Sakaguchi, T. Kurosaki, J. Burnside, K. Mirnics, and S. J. Corey. 2004. Microarray analysis of Lyn-deficient B cells reveals germinal center-associated nuclear protein and other genes associated with the lymphoid germinal center. *J. Immunol. 172:4133.*
33. Ren, C. L., T. Morio, S. M. Fu, and R. S. Geha. 1994. Signal transduction via CD40 involves activation of *lyn* kinase and phosphatidylinositol-3-kinase, and phosphorylation of phospholipase Cγ2. *J. Exp. Med. 179:673.*
34. Nishizumi, H., I. Taniuchi, Y. Yamanashi, D. Kitamura, D. Ilic, S. Mori, T. Watanabe, and T. Yamamoto. 1995. Impaired proliferation of peripheral B cells and indication of autoimmune disease in *lyn*-deficient mice. *Immunity 3:549.*
35. Kono, Y., K. Maeda, K. Kuwahara, H. Yamamoto, E. Miyamoto, K. Yonezawa, K. Takagi, and N. Sakaguchi. 2002. MCM3-binding GANP DNA-primase is associated with a novel phosphatase component G5PR. *Genes Cells 7:821.*

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Moriuchi M, and **Moriuchi H**.	YY1 transcription factor downregulates the expression of CCR5, a major coreceptor for HIV-1.	***J Biol Chem***	278	13003-13007	2003
Moriuchi M, Tamura A, and **Moriuchi H**.	In vitro reactivation of HIV-1 upon stimulation with scrub typhus rickettsial infection.	***Am J Trop Med Hyg***	68	557-561	2003
Moriuchi M, and **Moriuchi H**.	Increased susceptibility to HIV-1 of peripheral blood lymphocytes in acute infection with Epstein-Barr virus.	***J Med Virol***	71	343-346	2003
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
Moriuchi M, Yoshimine H, Oishi K, and **Moriuchi H**.	Norepinephrine inhibits human immunodeficiency virus type-1 infection through the NF-kappaB inactivation.	***Virology***	345	167-173	2006

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YY1 Transcription Factor Down-regulates Expression of CCR5, a Major Coreceptor for HIV-1*

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Expression of CCR5, a major coreceptor for human immunodeficiency virus type 1 (HIV-1), is regulated by a number of transcription factors. Here we report that the YY1 transcription factor down-regulates CCR5 promoter activity and that overexpression of YY1 reduces cell surface CCR5 expression and infectibility by R5-HIV-1. Because YY1 also down-regulates promoter activities of CXCR4, another major coreceptor for HIV-1 and HIV-1 long terminal repeat, this transcription factor may play a critical role in the pathogenesis of HIV-1 disease.

YY1 is a ubiquitous transcription factor that regulates a number of cellular and viral promoters, depending on the gene as well as the cell type in question (reviewed in Ref. 1). This transcription factor down-regulates expression from the human immunodeficiency virus type 1 (HIV-1)[1] long terminal repeat (2) as well as the promoter for CXCR4, a major coreceptor for X4-HIV-1 (3). Furthermore, YY1 may influence maturation and uncoating of the HIV-1 virions through its interaction with cyclophilin A (4). Thus, the YY1 transcription factor may play a critical role in the pathogenesis of HIV-1 disease.

CCR5, a receptor for the CC chemokines regulated upon activation, normal T cell-expressed and -secreted, macrophage inflammatory protein-1α and -1β is essential for cellular entry of R5-HIV-1 (reviewed in Ref. 5). Levels of its expression appear to be critical for infectibility by R5 HIV-1 (6) or rate of disease progression in infected individuals (7). We have cloned and characterized the promoter region of CCR5 and identified several transcription factors that transactivate it (8–10).

In this study, we demonstrate that YY1 down-regulates CCR5 expression, and overexpression of YY1 reduces cell surface CCR5 expression and infectibility by R5-HIV-1, further emphasizing its importance in the pathogenesis of HIV-1 disease.

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[1] The abbreviations used are: HIV, human immunodeficiency virus; GFP, green fluorescent protein; PBMC, peripheral blood mononuclear cells; IL-2, interleukin-2; PE, phycoerythrin; TSS, transcription start site.

EXPERIMENTAL PROCEDURES

Plasmids—Plasmids pGL-CCR5 and pGL-CCR5(−417) were described previously (8). Plasmid pGL-CCR5 (YY1-WT) contains the CCR5 promoter sequence spanning −607 to +61 relative to the major transcription start site (TSS) (8), followed by the luciferase reporter gene in plasmid pGL3-basic (Promega Corp., Madison, WI). Plasmid pGL-CCR5 (YY1-MT) has mutations (shown in bold letters below) at −594 and −593 relative to the TSS on the YY1 motif (indicated by underline) (AAAAAGATGGGAAA →AAAAAGATCCGAAA). Plasmids pCMV-YY1 and phPES2(−327/+59), a luciferase reporter driven by human cyclooxygenase-2 gene promoter, are generous gifts from T. Shenk (Princeton University, Princeton, NJ) (11) and H. Inoue (National Cardiovascular Center Research Institute, Osaka, Japan), respectively (12). To construct plasmid pcDNA/GFP-YY1, the YY1 coding region was amplified by polymerase chain reaction using forward primer 5′-ATGGCCTCGGGCGACACCCTCTAC-3′ and reverse primer 5′-TCACTGGTTGTTTTTGGCCTTAGC-3′, and cloned into pcDNA3.1/NT-GFP-TOPO (Invitrogen). The resultant plasmid expresses green fluorescence protein GFP-YY1 fusion protein under the control of the human cytomegalovirus major immediate early promoter. To construct pcDNA/GFP, pcDNA3.1/NT-GFP-TOPO was digested with *Kpn*I, treated with T4 DNA polymerase, and re-ligated with T4 DNA ligase.

Cells—Peripheral blood mononuclear cells (PBMC) were obtained from healthy volunteers (Red Cross Blood Center in Nagasaki Prefecture, Japan), and $CD4^+$ T cell-enriched PBMC were propagated as described previously (13). Where indicated, $CD4^+$ T cell-enriched PBMC were stimulated with recombinant human interleukin-2 (IL-2) (100 units/ml) for 7 days.

Transfection and Transient Expression Assays—Transfections of PBMC were performed using electroporation as described previously (14) or the Human T Cell Nucleofector™ kit (Amaxa Biosystems). In brief, 5×10^6 PBMC were resuspended in 100 μl of Human T Cell Nucleofector solution, mixed with a total of 5 μg of plasmid DNA, and pulsed using the Nucleofector program U-14. Luciferase assays for transient expression assays were performed as described previously (14). 293 cells were transfected as described previously (13).

Gel Mobility Shift Assays—Nuclear extracts were prepared from PBMC or 293 cells as described previously (15), and gel mobility shift assays were performed as described previously (15). Anti-YY1 rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Oligonucleotides used for competition studies are listed in Table I.

Flow Cytometric Analysis—Expression of cell surface CCR5 or CD4 was determined by staining cells with monoclonal anti-CCR5 Ab 2D7 phycoerythrin (PE)-conjugate or monoclonal anti-CD4 Ab RPA-T4 PE-conjugate (BD PharMingen), respectively, and analyzing in FACScan (Becton-Dickinson Immunocytometry Systems, San Jose, CA). GFP expression from pcDNA/GFP or pcDNA/GFP-YY1 was also analyzed in FACScan.

Single-round Viral Infection and Flow Cytometric Analysis for Intracellular Expression of p24 Ag—The recombinant, replication-incompetent virus NL4–3luc-R^-E^- that had been complemented with R5 JR-FL Env was designated NL4–3luc/JR-FL here and propagated as described previously (13). Approximately 5×10^6 PBMC were stimulated with hIL-2 (100 units/ml) for 7 days, transfected with 5 μg of pcDNA/GFP or pcDNA/GFP-YY1, and mock-infected or infected with NL4–3luc/JR-FL at 16 h after transfection.

For intracellular p24 Ag staining, the transfected and (mock-)infected cells were washed once in phosphate-buffered saline containing 5%

TABLE I
Oligonucleotides used for gel mobility shift assays
Sequences corresponding to cis-acting elements are underlined. Mutated nucleotides are shown in bold letters.

Oligonucleotide	Sequence
R5(−607/−584)	5′-TCCAGAAAAAGATGGGAAACCTGT-3′
R5(−607/−584)-MT	5′-TCCAGAAAAAGAT**CC**GAAACCTGT-3′
R5(−786/−763)	5′-TGAACTTGACCATATACTTATGTC-3′
YY1-C	5′-CGCTCCGCGGCCATCTTGGCGGCTGGT-3′
STAT-C	5′-GTATTTCCCAGAAAAGGAAC-3′

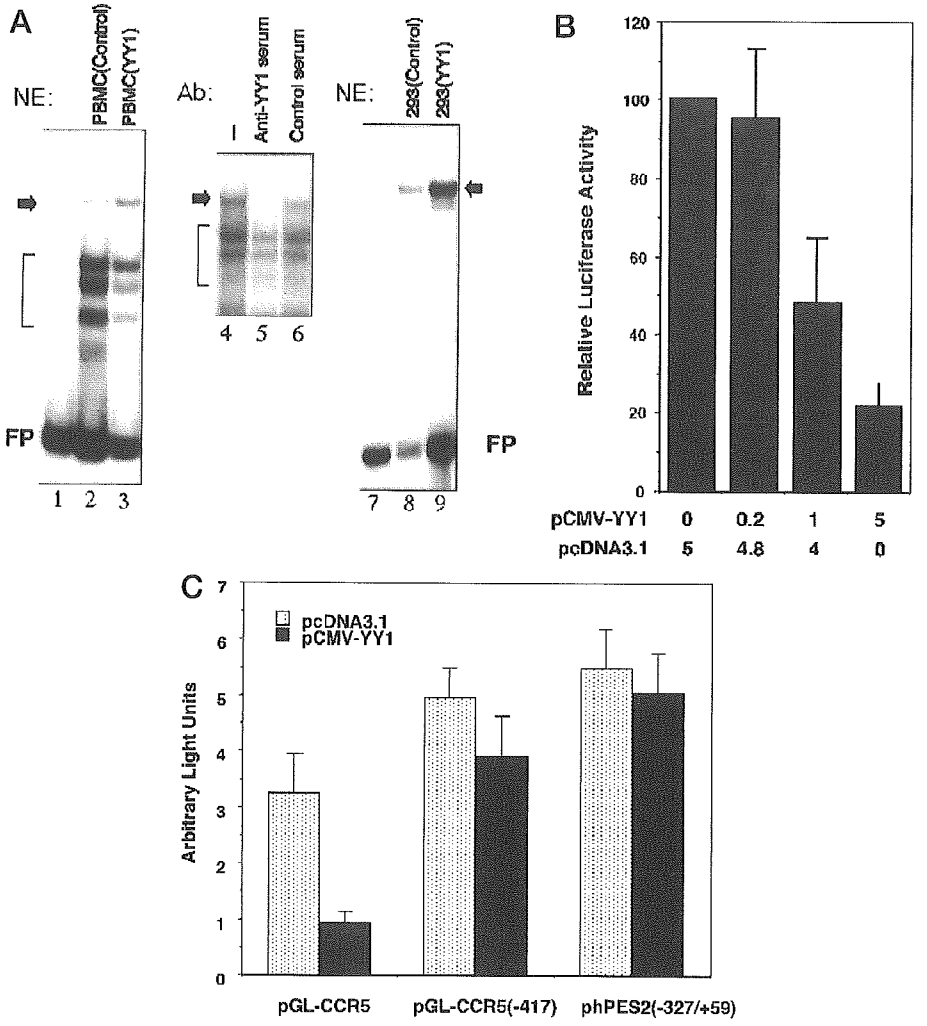

FIG. 1. **YY1 down-regulates CCR5 promoter.** *A*, nuclear extracts were prepared from 40×10^6 PBMC that had been transfected with pcDNA3.1 (*lane 2*) or pCMV-YY1 (*lanes 3–6*), or from 293 cells that had been transfected with pcDNA3.1 (*lane 8*) or pCMV-YY1 (*lane 9*). Gel mobility shift assay was performed with those nuclear extracts and YY1-C as a probe. *Lanes 1* and *7* represent probe alone. Where indicated, anti-YY1 rabbit polyclonal antibody (*lane 5*) or control rabbit serum (*lane 6*) was added to the reaction. *FP* indicates free probe. An *arrow* indicates the YY1 complex. A *bracket* indicates nonspecific complexes that appeared variably among experiments (data not shown) and were not disrupted by anti-YY1 antibody (*lane 5*). *B*, five million $CD4^+$ T cell-enriched PBMC were transfected with 2.5 μg of pGL-CCR5 along with the indicated amount of pCMV-YY1 and/or pcDNA3.1. Luciferase assays in the transfected cell lysates were performed 24 h after transfection. Reporter activity was shown as luciferase activity relative to that without pCMV-YY1 cotransfection. Results are reported as means ± S.E. from three independent experiments. *C*, five million $CD4^+$ T cell-enriched PBMC were transfected with 2.5 μg of pGL-CCR5, pGL-CCR5 (−417), or phPES2(−327/+59) along with 2.5 μg of pCMV-YY1 or pcDNA3.1. Luciferase activity was shown as arbitrary light units. Results are reported as means ± S.E. from three independent experiments.

male AB human serum (Sigma) and 0.04% sodium azide. The cells were fixed and permeabilized using the Cytofix/Cytoperm kit (BD PharMingen), washed twice with the wash buffer provided by the manufacturer, and resuspended in 50 μl of a 1:150 dilution of mouse anti-HIV-1 p24 monoclonal Ab PE conjugate (KC57; Coulter, Inc., Miami, FL). After a 30-min incubation in dark on ice, the cells were washed twice and analyzed in FACScan for GFP and p24 expression.

RESULTS

Overexpression of YY1 Down-regulates CCR5 Promoter—Because we and others have demonstrated that the YY1 transcription factor mediates a variety of effects on HIV-1 infection, we wanted to investigate whether this transcription factor plays a role in CCR5 expression on $CD4^+$ T cells, a target for HIV-1. First, we transfected $CD4^+$ T cell-enriched PBMC or 293 cells with pGL-CCR5 along with pCMV-YY1 or control plasmid. Gel mobility shift assay using YY1-C oligonucleotide (Table I) containing the YY1 consensus binding site demonstrated that the endogenous level of YY1 expression in PBMC or 293 cells was increased by transfection with pCMV-YY1 (Fig. 1*A*). Overexpression of YY1 down-regulated CCR5 promoter activity in a dose-dependent manner (Fig. 1*B*); however, truncation of the CCR5 promoter region down to −417 relative to the TSS resulted in much reduced responsiveness to YY1 (Fig. 1*C*), suggesting that a region upstream from −417 relative to the TSS contains a YY1-responsive element(s). In contrast, YY1 had little, if any, effect on cox-2 promoter activity (Fig. 1*C*).

Identification of a YY1 Binding Element on the CCR5 Promoter—By scanning the DNA sequence, we found two candidate sites for YY1 binding on the CCR5 promoter region upstream from −417 relative to the TSS. Gel mobility shift assays demonstrated that one of the two, spanning from −607 to −584 relative to the TSS, can form a DNA-protein complex that was disrupted by the YY1 consensus oligonucleotide (Fig. 2*A*, *lanes 7* and *8*). Unlabeled R5(−607/−584) oligonucleotide, but not R5(−607/−584)-MT in which the putative YY1 site was mutated (Table I), also disrupted the DNA-protein complex forma-

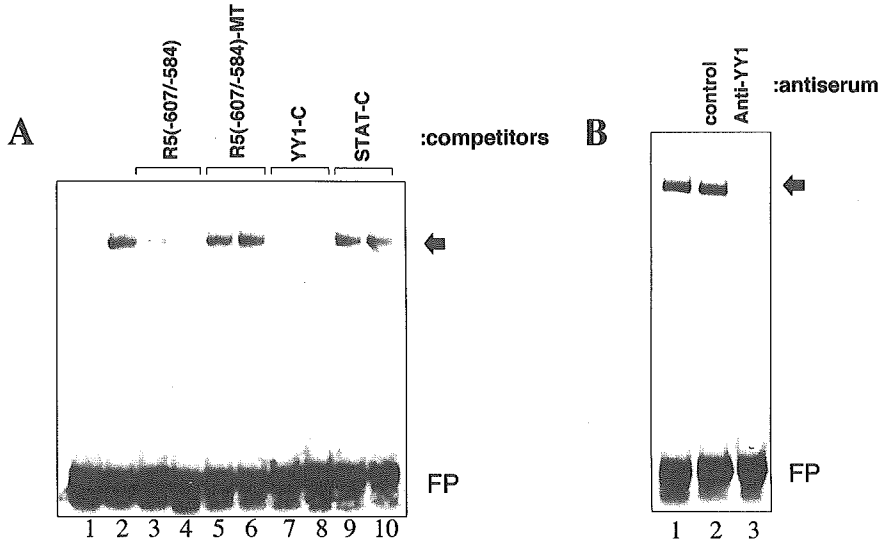

FIG. 2. **YY1 binds to CCR5 promoter.** *A*, gel mobility shift assays and competition studies. R5(−607/−584) probe was incubated with PBMC nuclear extracts in the presence of a 50- (*lanes 3, 5, 7,* and *9*) or 500-fold (*lanes 4, 6, 8,* and *10*) molar excess of non-labeled oligonucleotides (see Table I), indicated above the figure as competitors. *Lane 1* represents probe alone. *Lanes 2–10* represent reactions in the presence of PBMC nuclear extracts. *FP* indicates free probe. An *arrow* indicates the YY1 complex. *B*, gel-shift interference analysis with anti-YY1 antibody. The reaction mixture for binding between R5(−607/−584) oligonucleotide and PBMC extracts was incubated with either normal rabbit serum (*lane 2*) or anti-YY1 rabbit polyclonal antibody (*lane 3*). The *solid arrow* indicates the YY1 complex, which was disrupted by anti-YY1 antibody (*lane 3*), but not control serum (*lane 2*).

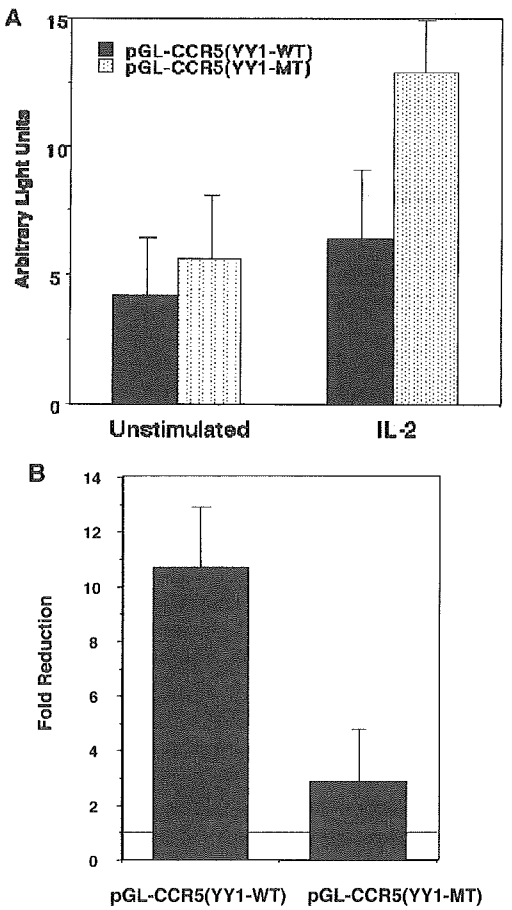

FIG. 3. **YY1 down-regulates CCR5 promoter.** *A*, effect of YY1 site mutation on CCR5 promoter activity. $CD4^+$ T cell-enriched PBMC were unstimulated or stimulated with IL-2 (100 units/ml) for 7 days and transfected with 2.5 μg of pGL-CCR5(*YY1-WT*) or pGL-CCR5(*YY1-MT*). Reporter activities are shown as arbitrary light units. Results are reported as means ± S.E. from three independent experiments. *B*, the YY1 motif on the CCR5 promoter mediates YY1-mediated transrepression. Five million $CD4^+$ T cell-enriched PBMC were transfected with 2.5 μg of pGL-CCR5(*YY1-WT*) or pGL-CCR5(*YY1-MT*) along with 2.5 μg of pCMV-YY1 or pcDNA3.1. *Fold reduction* indicates luciferase activity relative to basal promoter activity (pcDNA3.1 co-transfection). Results are reported as means ± S.E. from three independent experiments.

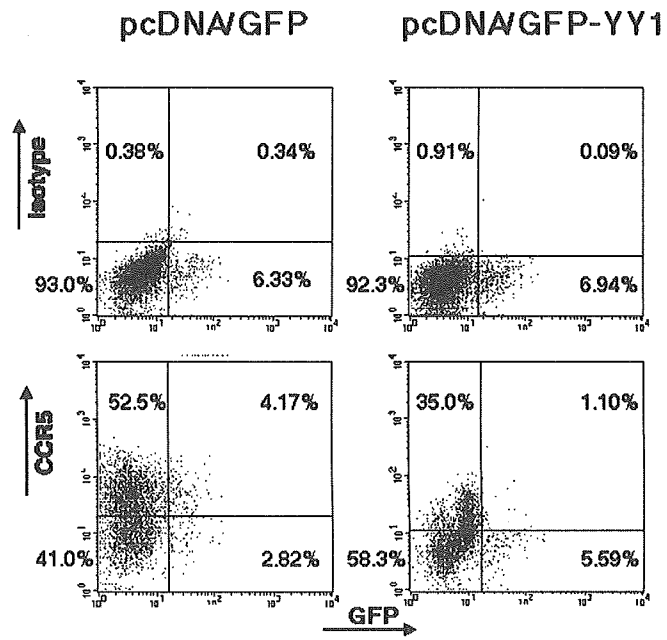

FIG. 4. **Overexpression of YY1 reduces cell surface expression of CCR5.** Five million $CD4^+$ T cell-enriched PBMC were stimulated with IL-2 for 7 days and transfected with 5 μg of pcDNA/GFP (*left panels*) or pcDNA/GFP-YY1 (*right panels*), stained with IgG2a PE (*upper panels*) or anti-CCR5 PE (*lower panels*), and analyzed in FACScan. The *dot blot* shows green fluorescence intensity (*GFP* or *GFP-YY1* expression) in *x*-axis and PE color intensity (*CCR5* expression) in *y*-axis. Results are representative of three independent experiments.

tion (Fig. 2*A*, *lanes 3–6*). Anti-YY1 antibody specifically disrupted DNA-protein complex formation (Fig. 2*B*, *lane 3*), indicating that this complex contains YY1. On the contrary, R5(−786/−763) probe, spanning from −786 to −763 relative to the TSS, could not form the YY1 complex (data not shown).

Mutation on the YY1 Binding Site Markedly Reduces YY1-mediated Down-regulation of CCR5 Promoter—To demonstrate that the aforementioned YY1 binding site is functional, YY1 effects on pGL-CCR5 (YY1-WT) or pGL-CCR5 (YY1-MT) in which the YY1 site is mutated were compared. Mutations on the YY1 site had minimal effect on basal activity of the CCR5 promoter but significantly inhibited CCR5 promoter activity induced by IL-2 stimulation (Fig. 3*A*) or phorbol myristate acetate plus ionomycin (data not shown). Thus, endogenous YY1 may play an important role in down-regulating the CCR5 promoter in cells that were stimulated to induce CCR5 expression, but not in unstimulated cells. YY1 potently down-regulated reporter activity of pGL-CCR5 (YY1-WT) by more than

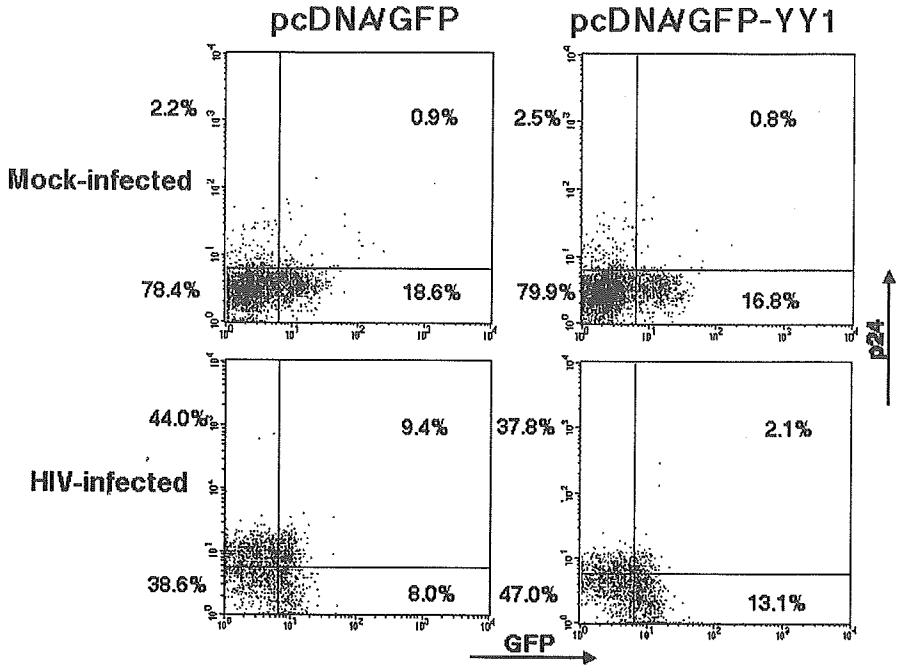

FIG. 5. **Overexpression of YY1 reduces infectibility of cells by R5-HIV-1.** $CD4^+$ T cell-enriched PBMC were stimulated with IL-2 for 7 days and transfected with pcDNA/GFP (*left panels*) or pcDNA/GFP-YY1 (*right panels*) and, 16 h later, mock-infected or infected with NL4–3luc/JR-FL. The cells were stained with anti-HIV-1 p24 Ab-PE or IgG1 PE (data not shown). The *dot blot* shows green fluorescence intensity (*GFP-YY1* expression) in *x*-axis and PE color intensity (*p24* expression) in *y*-axis. Results are representative of three independent experiments.

10-fold; however, mutations on the YY1 site markedly reduced YY1 suppression of reporter activity (less than 3-fold reduction) (Fig. 3*B*). These results suggest that the YY1 binding site is functional and plays a critical role in YY1-mediated down-regulation of the CCR5 promoter. Although residual responsiveness of pGL-CCR5 (YY1-MT) to YY1 may imply the presence of other YY1 binding site(s) on the promoter, we could not find any sequence resembling the YY1 motif.

Overexpression of YY1 Down-regulates Cell Surface Expression of CCR5—As shown above, the YY1 transcription factor can down-regulate the CCR5 promoter; however, it is well known that YY1 can mediate totally different effects, depending upon promoter constructs and cell types to be tested. To demonstrate whether cell surface expression of CCR5 is actually down-regulated by YY1, we overexpressed YY1 by transfecting $CD4^+$ T cell-enriched PBMC with pcDNA/GFP-YY1 and determined cell surface expression of CCR5 or CD4 by flow cytometry. Like pCMV-YY1, pcDNA/GFP-YY1 down-regulated reporter activity from pGL-CCR5 when co-transfected (data not shown). After a 7-day stimulation with IL-2, more than 30% of $CD4^+$ T cell-enriched PBMC expressed CCR5 (Fig. 4, *lower panels*; data not shown). GFP-positive cells expressed CCR5 at levels comparable with GFP-negative cells after pcDNA/GFP transfection (Fig. 4, *left panels*). However, significantly fewer GFP-positive cells expressed CCR5 after pcDNA/GFP-YY1 transfection (Fig. 4, *right panels*). Thus, overexpression of YY1 appears to reduce cell surface expression of CCR5. On the contrary, CD4 expression on GFP-positive cells after pcDNA/GFP transfection (94%; mean fluorescence intensity 36.9) was comparable with that on GFP-positive cells after pcDNA/GFP-YY1 transfection (93%; mean fluorescence intensity 34.0).

Overexpression of YY1 Decreased Infectibility by R5-HIV-1—Having had the aforementioned results, we wanted to determine whether overexpression of YY1 could inhibit HIV-1 infection. Because YY1 has variable effects depending upon cell type and because continuous and strong expression of YY1 is toxic to cells (data not shown), YY1 was transiently overexpressed in IL-2-stimulated, $CD4^+$ T cell-enriched PBMC by transfection with pcDNA/GFP-YY1. The transfected cells were then mock-infected or infected with NL4–3luc/JR-FL that is competent only for a single-round infection. HIV-1 infection was demonstrated by intracellular p24 Ag staining. As shown in Fig. 5, GFP-positive cells expressed p24 at levels comparable with GFP-negative cells after pcDNA/GFP transfection (Fig. 5, *left panels*). In contrast, many fewer GFP-positive cells expressed p24 after pcDNA/GFP-YY1 transfection (Fig. 5, *right panels*). Thus, overexpression of YY1 appeared to render $CD4^+$ T cells less infectible by R5-HIV-1.

Possible Anti-HIV-1 Activities Mediated by YY1

HIV-1 Replicative Cycle	YY1-Mediated effects
Entry	: Down-regulation of CCR5 / CXCR4 expression
↓	
Uncoating	: Inefficient uncoating due to lack of cyclophilin A in virion
↓	
Reverse Transcription / Nuclear Translocation / Integration	
↓	
Transcription	: Down-regulation of LTR activity
↓	
Translation	
↓	
Assembly / Maturation	: Block to incorporation of cyclophilin A into virion, leading to inefficient uncoating during the next cycle
↓	
Budding	

FIG. 6. **Possible anti-HIV-1 activities mediated by YY1.**

DISCUSSION

In this study we have demonstrated that the YY1 transcription factor can down-regulate expression of CCR5 at the promoter level. Levels of CCR5 expression appear to correlate well with infectibility of $CD4^+$ T cells by R5 HIV-1 (6) and rate of disease progression (7). Expression of CCR5 appears to be highly regulated by a number of cytokines, cell activation, or differentiation (16–20). At the promoter level, several transcription factors have been demonstrated to up-regulate CCR5 expression, including p65 (RelA) (21), C/EBP-β (22), GATA-1 (9), and Octamer (10). The present study has extended our understanding of the molecular mechanism of regulation of CCR5 promoter activity by adding YY1 as the first transcrip-

tional repressor of the promoter. We further demonstrated that overexpression of YY1 actually down-regulates cell surface CCR5 expression and infectibility by R5-HIV-1.

We have previously demonstrated that YY1 down-regulates the promoter for CXCR4, another major co-receptor for HIV-1 (3). YY1 is also known to down-regulate the HIV-1 long terminal repeat promoter (2). Furthermore, YY1 associates with cyclophilin A, which may be critical for maturation and uncoating of HIV-1 virions through its interaction with Gag (4). Taken together with the present study, it is reasonable to consider that the YY1 transcription factor plays important roles in the pathogenesis of HIV-1 disease (Fig. 6). Further investigations to delineate the molecular and cellular mechanisms that regulate expression of HIV coreceptors and to develop therapeutic interventions using anti-HIV-1 host factor(s) such as YY1 are warranted.

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REFERENCES

1. Shrivastava, A., and Calame, K. (1994) *Nucleic Acids Res.* **22,** 5151–5155
2. Margolis, D. M., Somasundaran, M., Green, M. R. (1994) *J. Virol.* **68,** 905–910
3. Moriuchi, M., Moriuchi, H., Margolis, D. M., and Fauci, A. S. (1999) *J. Immunol.* **162,** 5986–5992
4. Yang, W-.M., Inouye, C. J., and Seto, E. (1995) *J. Biol. Chem.* **270,** 15187–15193
5. Lusso, P. (2000) *Virology* **273,** 228–240
6. Wu, L., Paxton, W. A., Kassam, N., et al. (1997) *J. Exp. Med.* **185,** 1681–1691
7. Reynes, J., Portales, P., Segondy, M., Baillat, V., Andre, P., Avinens, O., Picot, M-.C., Clot, J., Eliaou, J-.F., and Corbeau, P. (2001) *AIDS* **15,** 1627–1634
8. Moriuchi, H., Moriuchi, M., and Fauci, A. S. (1997) *J. Immunol.* **159,** 5441–5449
9. Moriuchi, M., Moriuchi, H., and Fauci, A. S. (1999) *Blood* **93,** 1433–1435
10. Moriuchi, M., and Moriuchi, H. (2001) *J. Biol. Chem.* **276,** 8639–8642
11. Shi, Y., Seto, E., Chang, L-.S., and Shenk, Y. (1991) *Cell* **67,** 377–388
12. Inoue, H., and Tanabe T. (1998) *Biochem. Biophys. Res. Commun.* **244,** 143–148
13. Moriuchi, H., Moriuchi, M., and Fauci, A. S. (1998) *J. Exp. Med.* **187,** 1689–1698
14. Moriuchi, H., Moriuchi, M., and Fauci, A. S. (1997) *J. Immunol.* **158,** 3483–3491
15. Moriuchi, H., Moriuchi, M., and Cohen, J. I. (1995) *J. Virol.* **69,** 4693–4701
16. Bleul, C. C., Wu, L., Hoxie, J. A., Springer, T. A., and Mackay, C. R. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 1925–1929
17. Sozzani, S., Ghezzi, S., Iannolo, G., et al. (1998) *J. Exp. Med.* **187,** 439–444
18. Wang, J., Roderiquez, G., Oravecz, T., and Norcross, M. A. (1998) *J. Virol.* **72,** 7642–7647
19. Thivierge, M., Le Gouill, C., Tremblay, M. J., Stankova, J., and Rola-Pleszczynski, M. (1998) *Blood* **92,** 40–45
20. Tuttle, D. L., Harrison, J. K., Anders, C., Sleasman, J. W., and Goodenow, M. M. (1998) *J. Virol.* **72,** 4962–4969
21. Liu, R., Zhao, X., Gurney, T. A., and Landau, N. R. (1998) *AIDS Res. Hum. Retrovir.* **14,** 1509–1519
22. Rosati, M., Valentin, A., Patenaude, D. J., and Pavlakis, G. N. (2001) *J. Immunol.* **167,** 1654–1662

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IN VITRO REACTIVATION OF HUMAN IMMUNODEFICIENCY VIRUS-1 UPON STIMULATION WITH SCRUB TYPHUS RICKETTSIAL INFECTION

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Abstract. While a number of microbial infections induce a transient burst in viral load in individuals infected with human immunodeficiency virus-1 (HIV-1), a recent study has suggested that scrub typhus may suppress HIV-1 infection. We investigated the effects of *Orientia tsutsugamushi* on HIV-1 infection. *In vitro* HIV-1 infection experiments were conducted using peripheral blood mononuclear cells (PBMC) acutely infected with R5 and X4 HIV-1 or PBMC derived from patients receiving highly active antiretroviral therapy (HAART) whose plasma viral load was undetectable. Stimulation of PBMC with *O. tsutsugamushi* induced production of proinflammatory cytokines and β-chemokines, and markedly down-regulated expression of CCR5. Although pretreatment with *O. tsutsugamushi* rendered PBMC resistant to R5 HIV-1, it otherwise enhanced HIV-1 replication. Stimulation by *O. tsutsugamushi* induced HIV-1 replication in PBMC from patients receiving HAART. These findings suggest that scrub typhus does not necessarily suppress HIV-1 infection and does have potential to enhance HIV-1 replication.

INTRODUCTION

Immune activation or perturbation of cytokine networks following microbial coinfections generally increases human immunodeficiency virus-1 (HIV-1) viremia. However, Watt and others have recently reported the possible HIV-1-inhibitory effects of scrub typhus,[1] which is an acute febrile disease that is endemic in tropical Asia where HIV-1 infection is also prevalent. Their hypothesis is intriguing but also remains somehow speculative, partly because of difficulty in conducting a prospective and well-controlled clinical study in Thailand, as well as a lack of precise laboratory data supporting their hypothesis. We investigated effects of *Orientia tsutsugamushi* infection on *in vitro* HIV-1 infection of peripheral blood mononuclear cells (PBMC).

MATERIALS AND METHODS

Cells. The PBMC were isolated from healthy volunteers who were not infected with HIV, as described previously.[2] Resting $CD4^+$ T cells were isolated from HIV-1-infected individuals whose plasma viral RNA was undetectable upon receiving highly active antiretroviral therapy (HAART), as described previously.[3,4]

Viruses and rickettsiae. Virus stocks were prepared by transfecting 293 cells with the following HIV-1 infectious molecular clones:[2] AD8 (subtype B, R5), YU-2 (subtype B, R5), NL4-3 (subtype B, X4), ELI1 (subtype B, X4), 89.6 (subtype B, R5X4), and 93JP-NH1 (subtype E, R5X4). The proinflammatory cytokines tumor necrosis factor- α (TNF-α) and interleukin-1β (IL-1β), as well as the β-chemokines regulated upon activation normal T cell expressed and secreted (RANTES) protein and macrophage inflammatory protein-1β, were not detected in these virus stocks.

Cell-free stocks of *O. tsutsugamushi* strains Karp or Kuroki were propagated in L929 cells.[5] In certain experiments, *O. tsutsugamushi* stocks were pretreated at 56°C for 60 minutes. Rickettsial replication was not detected by the L929 cell culture method.

Infections. For acute infection experiments, PBMC derived from healthy volunteers were mock-infected or infected with cell-free stocks of *O. tsutsugamushi* strains Karp or Kuroki, and then infected with HIV-1 stocks. Inocula were standardized by reverse transcriptase (RT) activity (10,000 cpm per 4×10^5 cells, as determined by RT assays). Viral replication was monitored by RT activity in cell-free culture supernatants.[2] For reactivation experiments, PBMC derived from patients receiving HAART were mock-infected or infected with the Karp strain, and viral replication was monitored by measuring p24 antigen levels in cell-free culture supernatants.[3,4]

Single-round viral replication assays. A replication-incompetent luciferase-reporter recombinant virus NL4-3luc-R^-E^- was pseudotyped with envelope (env) protein from R5 HIV-1 JRFL or X4 HIV-1 HXB2.[2] The PBMC were not treated, pretreated, or post-treated with the Karp strain (live or heat-inactivated) or supernatants from *O. tsutsugamushi* (Karp strain)-infected autologous PBMC, and luciferase activity in the infected cell lysates was determined.[2]

Flow cytometry. Cell surface expression of CCR5 and CXCR4 was demonstrated by staining cells with phycoerythrin-conjugated anti-CCR5 monoclonal antibody 2D7 and anti-CXCR4 monoclonal antibody 12G5 (R&D Systems, Minneapolis, MN) and analyzed by FACScan (Becton-Dickinson Immunocytochemistry Systems; San Jose, CA).[2]

Transient expression assays. Forty million PBMC were transfected with 40 μg of pGL-HIV-1-LTR (a luciferase reporter under the control of the HIV-1 long terminal repeat [LTR]) along with 10 μg of pSV2-CAT or pSV2-Tat (encoding HIV-1 Tat), and luciferase activity in the transfected cells was determined, as described previously.[2]

Enzyme-linked immunosorbent assay (ELISA). Levels of cytokines were determined by commercially available ELISA kits, according to the manufacturer's instruction (R&D Systems).

RESULTS

Dichotomous effects of *O. tsutsugamushi* on *in vitro* HIV-1 infection. In acute HIV-1 infection experiments, PBMC were infected with *O. tsutsugamushi*, and then infected with R5 HIV-1 (which uses chemokine receptor CCR5 to infect the cell), X4 HIV-1 (which uses CXCR4), or R5X4 HIV-1 (which uses both CCR5 and CXCR4). Stimulation with *O. tsutsugamushi* consistently enhanced infection with X4 or R5X4 HIV-1, irrespective of subtypes B or E (Figure

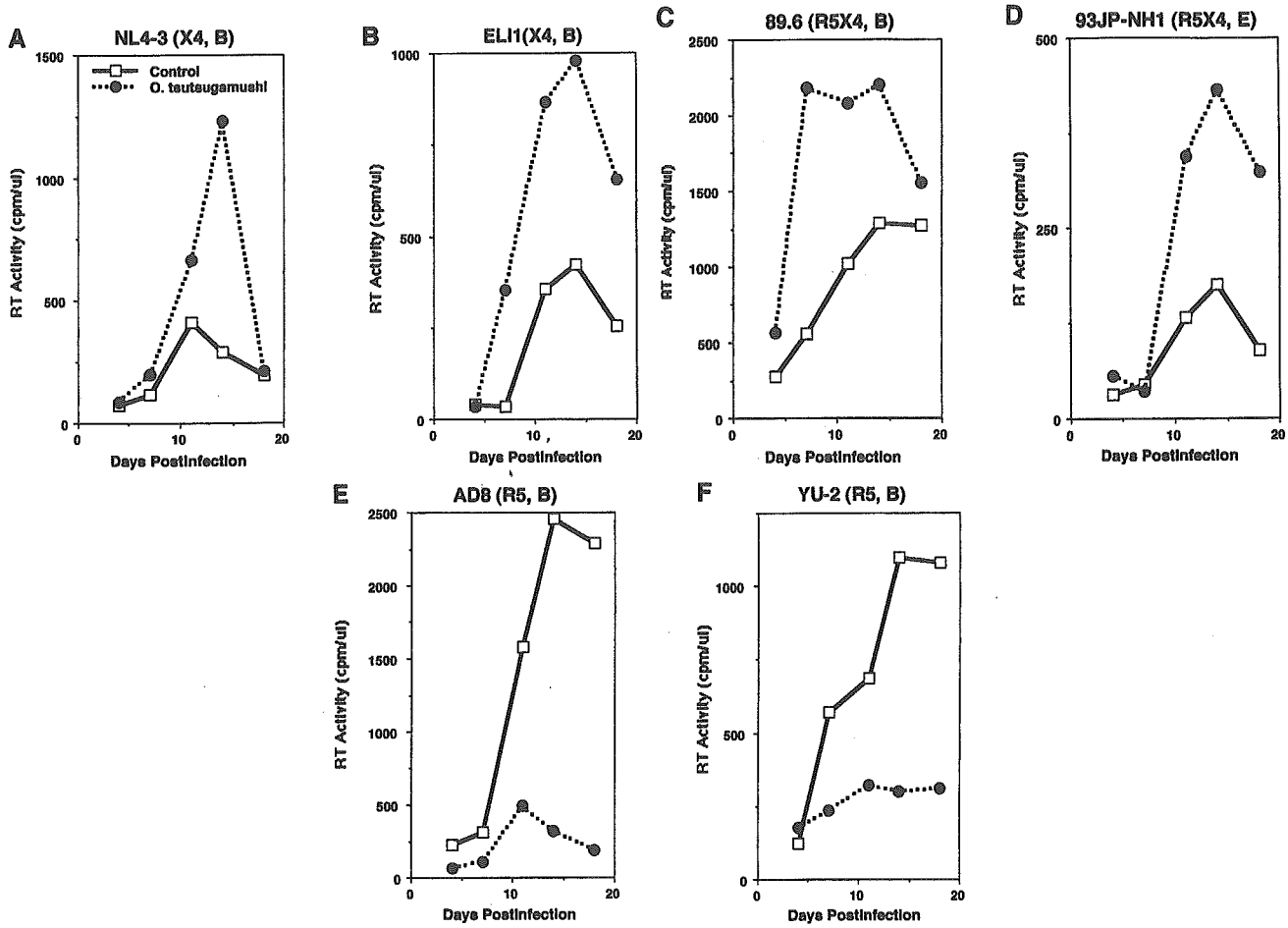

FIGURE 1. Dichotomous effects of *Orientia tsutsugamushi* on *in vitro* replication of human immunodeficiency virus-1 (HIV-1). Infection with *O. tsutsugamushi* enhanced replication of X4 HIV-1 (**A**, NL4-3 and **B**, ELI1) and R5X4 HIV-1 (**C**, 89.6 and **D**, 93JP-NH1), but suppressed replication of R5 HIV-1 (**E**, AD8 and **F**, YU-2). Peripheral blood mononuclear cells were uninfected (**squares**) or infected (**circles**) with the *O. tsutsugamushi* Karp strain for 24 hours prior to HIV-1 infection, and HIV-1 replication was monitored by reverse transcriptase (RT) assays. Similar results were obtained using the Kuroki strain of *O. tsutsugamushi*.

1A–D). However, the R5 HIV-1-inhibitory effect of *O. tsutsugamushi* was not consistent; it either suppressed (Figure 1E and F and donors 1, 3, and 5 in Table 1) or enhanced (donors 2 and 4 in Table 1) replication of R5 HIV-1. Cell viability of *O. tsutsugamushi*-stimulated cultures, as judged by trypan blue staining, was similar to that of unstimulated cultures. These results suggested that *O. tsutsugamushi* has several different activities against HIV-1 infection.

To more precisely delineate how *O. tsutsugamushi* can modulate HIV-1 infection, we performed single-round viral replication assays in which PBMC were uninfected or infected with *O. tsutsugamushi* prior to or after HIV-1 infection. In these assays, pretreatment with *O. tsutsugamushi* suppressed R5, but not X4 HIV-1 infection (Figure 2). In contrast, posttreatment enhanced HIV-1 infection, irrespective of coreceptor usage (Figure 2). Heat-inactivated *O. tsutsugamushi* had effects similar to those of live *O. tsutsugamushi* stocks (Figure 2). Thus, *O. tsutsugamushi* appeared to inhibit CCR5-mediated cellular entry of R5 HIV-1 and facilitate the post-entry viral replicative cycle. In addition, the productive replication of *O. tsutsugamushi* is not necessary for its effects.

Down-regulation of CCR5 expression by *O. tsutsugamushi*. Since the level of CCR5 expression correlates well with infectability of cells with R5 HIV-1, we hypothesized that *O. tsutsugamushi* infection leads to down-regulation of CCR5 expression. Flow cytometry studies have clearly proven our hypothesis: *O. tsutsugamushi* infection down-regulated CCR5 expression (Figure 3 and Table 1) but had little effect on CXCR4 expression.[6]

Up-regulation of HIV-1 LTR activity by *O. tsutsugamushi*. Next, we investigated how *O. tsutsugamushi* infection en-

TABLE 1

CCR5 expression and infectability by R5 human immunodeficiency virus-1 (HIV-1) of peripheral blood mononuclear cells (PBMC) stimulated with *Orientia tsutsugamushi**

	CCR5 expression (%)		R5 HIV-1 infectability (cpm/μL)	
Donor	Unstimulated	Stimulated	Unstimulated	Stimulated
1	5.1	<0.5	2,460	480
2	6.8	1.1	1,980	4,230
3	2.4	<0.5	2,690	140
4	0.8	<0.5	120	710
5	3.6	<0.5	2,360	220

* Cell surface CCR5 expression was determined by flow cytometry 24 hours after stimulation of PBMC from five donors with the *O. tsutsugamushi* Karp strain. Peak reverse transcriptase titers in infection of unstimulated or stimulated PBMC with HIV-1 AD8 are shown as R5 HIV-1 infectability. Results in donor 1 are also shown in Figures 1E and 3.

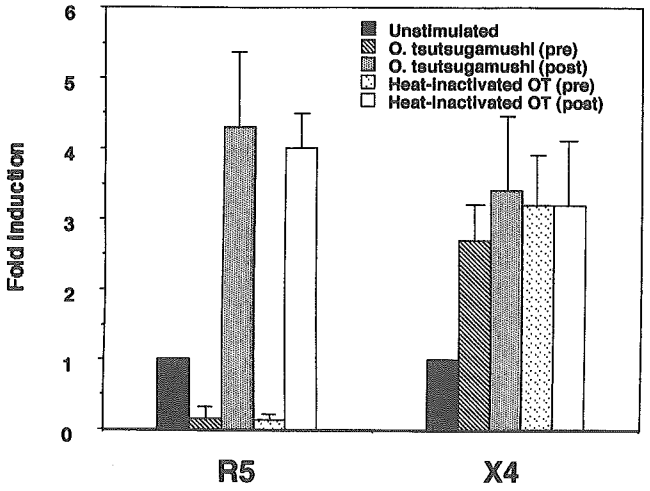

FIGURE 2. Differential effects of *Orientia tsutsugamushi* (OT) on R5 human immunodeficiency virus-1 (HIV-1) infection before and after cellular entry. Pretreatment with *O. tsutsugamushi* suppressed R5 HIV-1 infection, while post-treatment enhanced HIV-1 infection. A replication-incompetent luciferase-reporter recombinant virus pseudotyped with envelope (env) protein from R5 HIV-1 JRFL or X4 HIV-1 HXB2 was infected into peripheral blood mononuclear cells (PBMC). The PBMC were not treated, pretreated (pre), or post-treated (post) with the *O. tsutsugamushi* Karp strain (live or heat-inactivated), and luciferase activity in the infected cell lysates was determined. Fold induction indicates luciferase activity relative to that of a control (unstimulated) experiment. Results (mean ± SD) of triplicate experiments are shown.

hanced post-entry HIV-1 replication. In transient expression assays using PBMC, HIV-1 LTR promoter activity from pGL-HIV-1-LTR was markedly enhanced by stimulation with *O. tsutsugamushi* infection (Figure 4). Thus, increased viral replication resulted, at least in part, from up-regulation of viral transcription.

Mediation of the effects of *O. tsutsugamushi* infection on HIV-1 infection through soluble activity. In good agreement with previous studies demonstrating that *O. tsutsugamushi* infection induces production of a number of cytokines including TNF-α, interferon-γ (IFN-γ), and β-chemokines,[7,8] levels of TNF-α, IFN-γ, and RANTES protein in cell-free supernatants of *O. tsutsugamushi*-infected PBMC were 645 pg/mL, 170 pg/mL, and 8,350 pg/mL, respectively, while uninfected cells released < 15.6 pg/mL of TNF-α, <15.6 pg/mL of IFN-γ, and 120 pg/mL of RANTES protein, respectively. Although proinflammatory cytokines such as TNF-α have been shown to transactivate HIV-1 LTR activity, IFN-γ and RANTES protein have dichotomous effects on HIV-1 replication, depending upon cell types and/or virus strains.[2] To demonstrate that soluble activity mediated by those cytokines plays a critical role in modulating HIV-1 infection of *O. tsutsugamushi*-infected PBMC, cell-free supernatants were collected from *O. tsutsugamushi*-infected PBMC. Autologous uninfected PBMC were treated with the supernatants along with minocycline (5 μg/mL) to prevent replication of *O. tsutsugamushi* prior to or after infection with HIV-1. Minocycline did not influence HIV-1 infection at the concentration used in this study. As shown in Figure 5, soluble factors derived from *O. tsutsugamushi*-infected cells had similar effects on HIV-1 infection to *O. tsutsugamushi* infection *per se*.

***In vitro* reactivation of HIV-1 upon stimulation with *O. tsutsugamushi*.** Finally, we tested whether stimulation with *O. tsutsugamushi* can induce HIV-1 replication from PBMC derived from HIV-1-infected individuals whose plasma viral RNA was undetectable after HAART. Apheresis to obtain PBMC was performed according to protocol approved by the National Institute of Allergy and Infectious Diseases Institutional Review Board (Bethesda, MD). The $CD8^+$ T cells and $HLA\text{-}DR^+$ cells were depleted by Dynabeads (Dynal, Lake Success, NY) CD8 and HLA-DR, and there were less than 0.1% $HLA\text{-}DR^+CD4^+$ T cells (activated $CD4^+$ T cells) in the remaining PBMC preparations. These cells did not release HIV-1 p24 antigen without any stimulation; however, stimulation with phytohemagglutinin plus IL-2 induced *in vitro* HIV-1 replication in all patients tested (Table 2). When these cells were stimulated with *O. tsutsugamushi*, HIV-1 replication was induced in two of the four patients tested (Table 2). Thus, *O. tsutsugamushi* stimulation appears to have capability of inducing HIV-1 replication in patients whose plasma viremia is undetectable.

DISCUSSION

Orientia tsutsugamushi is a gram-negative bacillus that is an obligate intracellular parasite, and is distributed in many Asian countries and Pacific islands. Since HIV-1 infection is

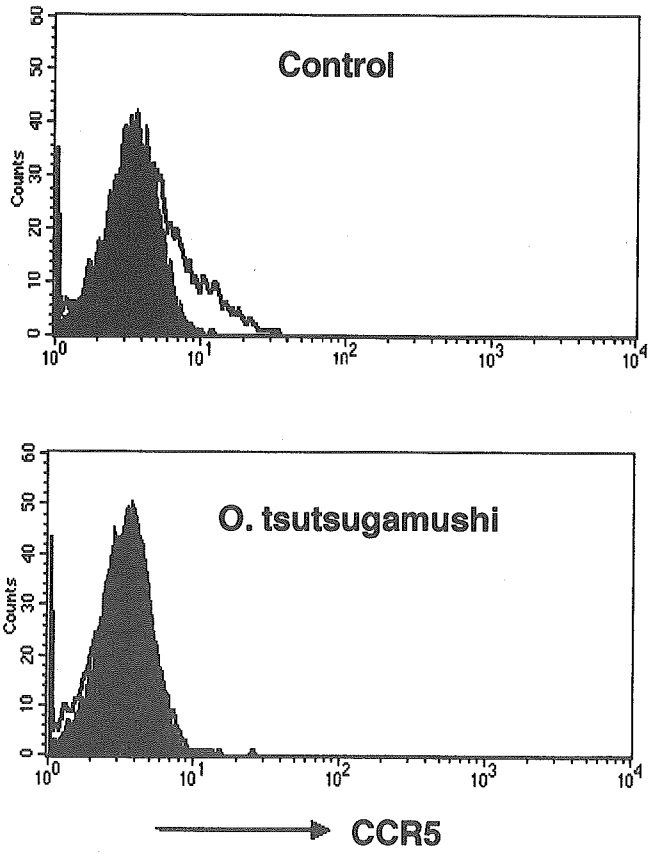

FIGURE 3. Down-regulation of expression of CCR5 by infection with *Orientia tsutsugamushi*. Peripheral blood mononuclear cells were infected with the Karp strain, and 24 hours later cell-surface expression of CCR5 was determined by flow cytometry using a monoclonal antibody (2D7) to CCR5. Staining with an isotype control (**shaded areas**) and with the monoclonal antibody to CCR5 (**solid lines**) are shown.

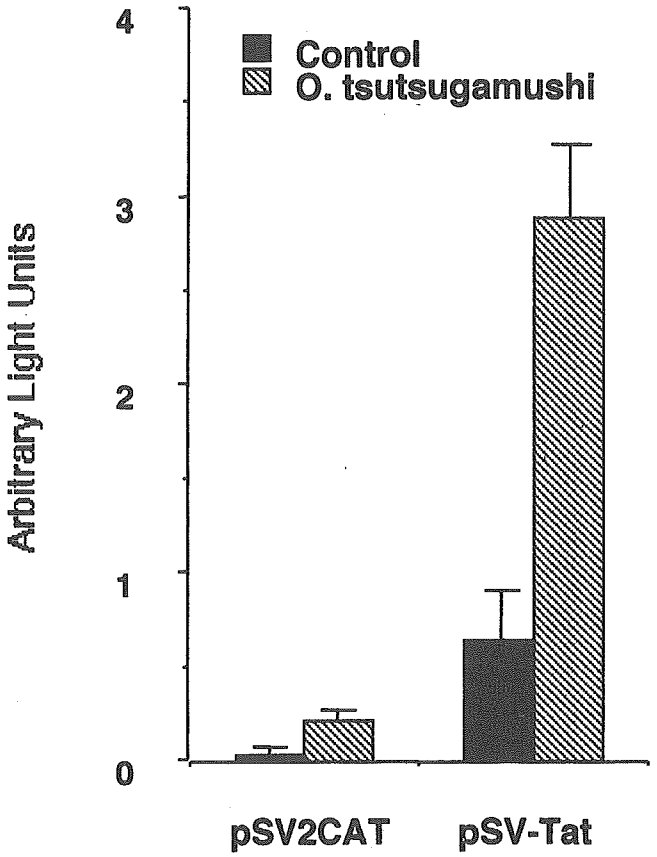

FIGURE 4. Up-regulation of human immunodeficiency virus-1 (HIV-1) long terminal repeat (LTR) activity by *Orientia tsutsugamushi*. Peripheral blood mononuclear cells were transfected with pGL-HIV-1-LTR along with pSV2CAT or pSV-Tat, and stimulated with *O. tsutsugamushi*. Luciferase activity in the transfected cell lysates was determined two days after transfection. Results (mean ± SD) of triplicate experiments are shown.

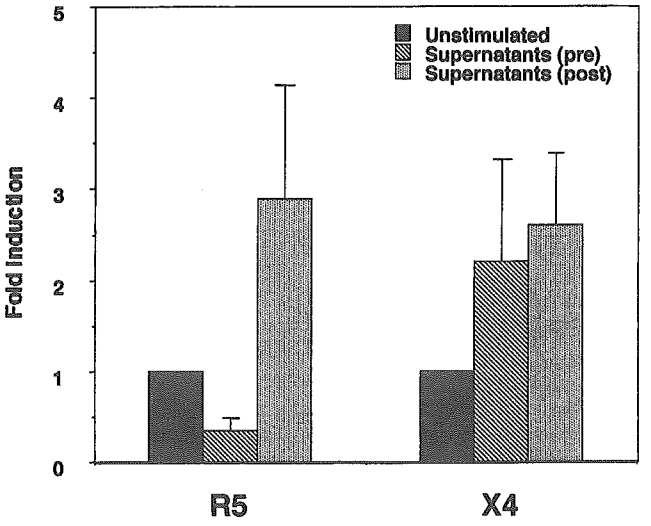

FIGURE 5. Mediation of the effects of infection with *Orientia tsutsugamushi* on human immunodeficiency virus-1 (HIV-1) infection through soluble activity. Peripheral blood mononuclear cells (PBMC) were infected with a replication-recombinant virus pseudotyped with envelope (env) protein from R5 HIV-1 JRFL or X4 HIV-1 HXB2. The PBMC were not treated, pretreated (pre), or post-treated (post) with supernatants from autologous PBMC infected with the *O. tsutsugamushi* Karp strain, and luciferase activity in the cell lysates was determined. Fold induction indicates luciferase activity relative to that of a control (untreated) experiment. Results (mean ± SD) of triplicate experiments are shown.

also endemic in some of these areas, interaction between the two microorganisms may have clinical significance. In this regard, a recent study from Thailand has reported that acute scrub typhus infection may suppress HIV-1 infection,[1] which is in striking contrast to many other microbial coinfections that generally result in enhanced replication of HIV-1.[9] However, difficulty in conducting a prospective and well-controlled clinical study in Thailand, as well as the lack of precise laboratory data supporting their hypothesis, has resulted in substantial controversy.

Our *in vitro* study has suggested that *O. tsutsugamushi* infection had impacts on HIV-1 infection in several different ways, and that the net effect depends upon the balance of positive and negative factors. Difference in the net effects observed among different donors is intriguing and deserves further evaluation. Our preliminary data suggest that a number of host factors are involved in such variability (Moriuchi M, Moriuchi H, unpublished data). For example, while up-regulation of HIV-1 expression from the LTR promoter secondary to proinflammatory cytokine production would benefit HIV-1 infection, down-regulation of CCR5 expression as well as production of chemokines would inhibit HIV-1 entry. Activation of LTR by microbial stimulation has been demonstrated in a variety of other co-infections.[9] We have previously reported similar dichotomous effects of bacterial cell wall components such as lipopolysaccharide (LPS) of gram-negative bacilli, lipoteichoic acid of gram-positive cocci, and lipoarabinomannan of mycobacteria on HIV-1 infection.[2] Our results in the present study were not unexpected, since *O. tsutsugamushi* contains LPS in its cell wall structure, although rickettsial LPS generally has weaker endotoxin activity than that of other gram-negative bacilli. Thus, although it is still possible that *in vivo* infection with *O. tsutsugamushi* may have substantially distinct effects on HIV-1 infection, our *in vitro* study suggests that scrub typhus does not necessarily suppress HIV-1 infection and does have potential to enhance HIV-1 replication.

Interactions between HIV and other microbes do not appear to be simple. Recently, measles infection has been shown to suppress HIV viremia in patients,[10] and we have also demonstrated that malaria parasites may suppress HIV-1 infec-

TABLE 2

In vitro reactivation of human immunodeficiency virus-1 (HIV-1) upon stimulation with *Orientia tsutsugamushi**

Patient	CD4+ T cells (/μL)	HIV-1 RNA copies (/mL)	p24 (pg/mL)		
			Unstimulated	*O. tsutsugamushi*	PHA/IL-2
1	303	<50	<7.8	<7.8	54
2	238	<500	<7.8	27	55
3	601	<500	<7.8	8.0†	116
4	596	<50	<7.8	39	64

Peripheral blood mononuclear cells were derived from patients 1 through 4 3,4 whose plasma viremia was undetectable by the Amplicor Ultrasensitive HIV-1 Monitor assay (Roche, Diagnostics Corporation, Indianapolis, IN) in patients 1 and 4 or bDNA assays (Chiron, Emeryville, CA) in patients 2 and 3, were depleted of $CD8^+$ cells and $HLA\text{-}DR^+$ cells, and were either unstimulated or stimulated as indicated. Peak HIV-1 p24 titers that were obtained on day 16 are shown. PHA = phytohemagglutinin, IL-2 = interleukin-2.

† HIV-1 replication in the PBMC of patient 3, as determined by slight increase in p24 titer on day 16, was not able to be expanded by further passages.

tion *in vitro*.[11] Further studies are required for better understanding of interaction between these microbes and HIV-1 in co-infected patients.

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REFERENCES

1. Watt G, Kantipong P, de Souza M, Chanbancherd P, Jongsakul K, Ruangweerayud R, Loomis-Price LD, Polonis V, Myint KS, Birx DL, Brown AE, Krishna S, 2000. HIV-1 suppression during acute scrub-typhus infection. *Lancet 356:* 475–479.
2. Moriuchi M, Moriuchi H, Turner W, Fauci AS, 1998. Exposure to bacterial products renders macrophages highly susceptible to T-tropic human immunodeficiency virus type 1: implications for *in vivo* coinfections. *J Clin Invest 102:* 1540–1550.
3. Moriuchi H, Moriuchi M, Mizell SB, Ehler LA, Fauci AS, 2000. *In vitro* reactivation of HIV-1 from latently infected, resting CD4+ T cells upon bacterial stimulation. *J Infect Dis 181:* 2041–2044.
4. Moriuchi H, Moriuchi M, 2000. *In vitro* induction of HIV-1 replication in resting $CD4^+$ T cells derived from individuals with undetectable plasma viremia upon stimulation with human T-cell leukemia virus type I. *Virology 278:* 514–519.
5. Tamura A, Urakami H, Tsuruhara T, 1988. Purification of *Rickettsia tsutsugamushi* by Percoll density gradient centrifugation. *Microbiol Immunol 26:* 321–328.
6. Shalekoff S, Tiemessen CT, 2001. Duration of sample storage dramatically alters expression of the human immunodeficiency virus coreceptors CXCR4 and CCR5. *Clin Diagn Lab Immunol 8:* 432–436.
7. Iwasaki H, Takada N, Nakamura T, Ueda T, 1997. Increased levels of macrophage colony-stimulating factor, gamma interferon, and tumor necrosis factor alpha in sera of patients with *Orientia tsutsugamushi* infection. *J Clin Microbiol 35:* 3320–3322.
8. Cho NH, Seong SY, Huh MS, Han TH, Koh YS, Choi MS, Kim IS, 2000. Expression of chemokine genes in murine macrophages infected with *Orientia tsutsugamushi. Infect Immun 68:* 594–600.
9. Blanchard A, Montagnier L, Gougeon ML, 1997. Influence of microbial infections on the progression of HIV disease. *Trends Microbiol 5:* 326–331.
10. Moss WJ, Ryon JJ, Monze M, Cutts F, Quinn TC, Griffin DE, 2002. Suppression of human immunodeficiency virus replication during acute measles. *J Infect Dis 185:* 1035–1042.
11. Moriuchi M, Moriuchi H, Mon HM, Kanbara H, Dichotomous effects of *Plasmodium falciparum* antigens on expression of human immunodeficiency virus (HIV) co-receptors on and infectability by HIV of CD4+ T cells. *J Infect Dis 186:* 1194–1197.

Journal of Medical Virology 71:343–346 (2003)

Increased Susceptibility to HIV-1 of Peripheral Blood Lymphocytes in Acute Infection With Epstein-Barr Virus

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Epstein-Barr virus (EBV) is an important pathogen in human immunodeficiency virus (HIV)-infected individuals that causes lymphoma and other lymphoproliferative disorders upon disease progression; however, interaction between the two viruses during acute infection is not well known. Expression of CCR5, a major coreceptor for HIV, was enhanced on $CD4^+$ T cells from patients with acute EBV infection. Furthermore, susceptibility of those cells to R5-HIV-1, but not X4-HIV-1, was increased. EBV effects on CCR5 expression on or susceptibility to R5-HIV-1 of $CD4^+$ T cells did not require coinfection of the same cell with the two viruses, because $CD4^+$ T cells from patients with acute EBV infection were not infected with EBV. Considering that both HIV and EBV are transmitted by intimate contact, such possible interaction between the two viruses may have implications for viral transmission and the pathogenesis of HIV disease. ***J. Med. Virol. 71:343–346, 2003.***

KEY WORDS: $CD4^+$ T cells; CCR5; viral transmission

INTRODUCTION

Chemokine receptor CCR5 serves as a major coreceptor for HIV-1, and levels of $CD4^+$ T cell surface expression correlate well with in vitro susceptibility to R5-HIV-1 [Wu et al., 1997] and determine the rate of $CD4^+$ T cell decline or HIV-1 disease progression in patients [Reynes et al., 2001]. CCR5 expression is variable among individuals and is regulated by immunologic [Moriuchi et al., 1997, 1999; Patterson et al., 1999; Moriuchi and Moriuchi, 2001, 2003] or microbial [Moriuchi et al., 1998a,b; Juffermans et al., 2000; Tkachuk et al., 2001] stimulation. It has been shown that CCR5 is expressed characteristically on type 1 T helper cells (Th1s) [Bonecchi et al., 1998] that control predominantly cell-mediated immune responses and appear to be involved in chronic inflammatory conditions.

Epstein-Barr virus (EBV) infects B cells and acute EBV infection causes infectious mononucleosis (IM), in which a large number of $CD4^+$ T cells as well as $CD8^+$ T cells are activated. Not surprisingly, these T cells exhibit Th1 phenotypes [Zaunders et al., 2001] to prevent from malignant transformation of infected B cells. Therefore, we hypothesized that $CCR5^+CD4^+$ T cells are increased and support R5 HIV-1 infection in acute EBV infection. To test this hypothesis, we tried to determine CCR5 expression levels and in vitro susceptibility to R5 HIV-1 of $CD4^+$ T cells derived from patients with IM.

MATERIALS AND METHODS

Patients and Cells

Six patients (6–28 years; mean age, 16 years) with IM were recruited and tested for EBV antibodies. Acute EBV infection was confirmed by the presence of IgM antibody to viral capsid antigen (VCA) or seroconversion of anti-VCA IgG. Informed consent was obtained from individuals with demonstrated acute EBV infection or their parents, and heparinized blood was drawn at acute phase (within 7 days after onset of the disease) and convalescent phase (3–4 weeks after onset of the disease). Peripheral blood mononuclear cells (PBMC) were isolated from the patients or healthy control individuals, and $CD4^+$ T cells were purified (>98%) as described previously [Moriuchi et al., 2000a,b] and

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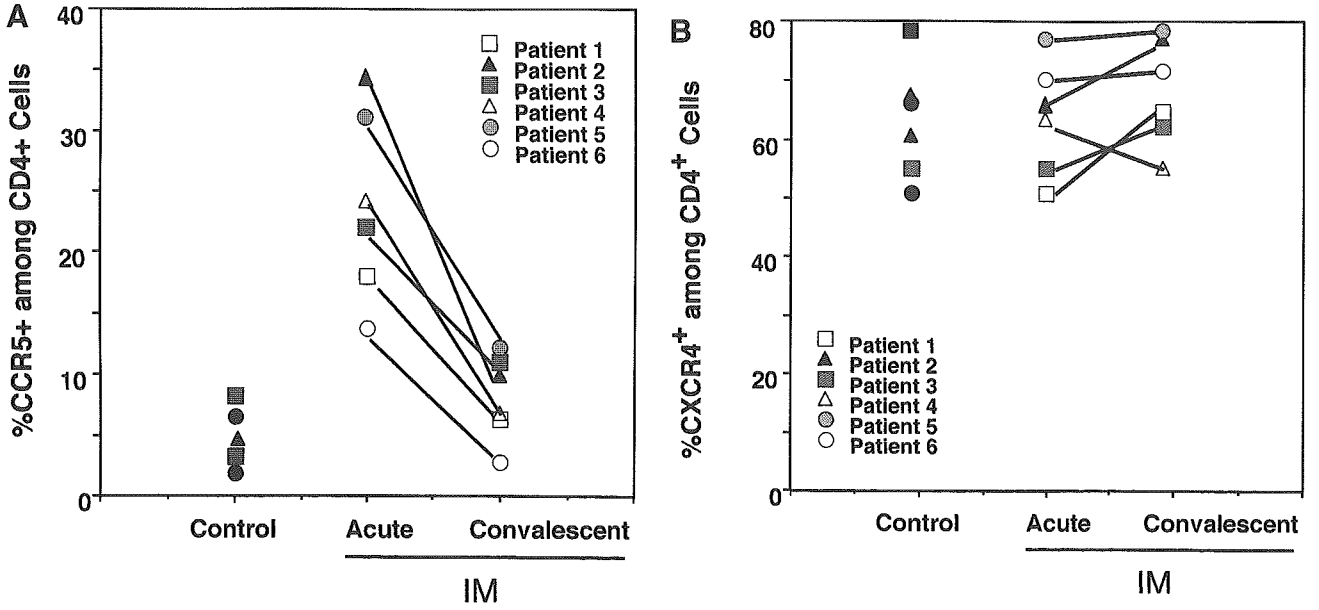

Fig. 1. Expression of CCR5 (**A**) or CXCR4 (**B**) on $CD4^+$ T cells derived from 6 patients with IM and 6 healthy control individuals. Each pair of acute and convalescent phases of EBV infection are connected by solid lines. Percentage of $CCR5^+$ cells (A) or $CXCR4^+$ cells (B) among $CD4^+$ T cells is shown.

frozen at −80°C until use for infection experiments. Approximately 3×10^5 $CD4^+$ T cells from each donor were tested for EBV DNA and HIV-1 proviral DNA with polymerase chain reaction, and neither of them was detected (<10 copies).

Flow Cytometry

Monoclonal antibodies (MAb), CD4-fluorescein isothiocyanate (FITC) (clone RPA-T4), CCR5-phycoerythrin (PE) (clone 2D7), and CXCR4-PE (clone 12G5), were purchased from BD PharMingen (San Diego, CA). Approximately 300 μl fresh heparinized blood was incubated for 30 min at 4°C in the dark with 8 μg/ml anti-CD4-FITC MAb, and 8 μg/ml anti-CCR5-PE or anti-CXCR4-PE MAb. Isotype-matched MAb controls (BD PharMingen), used at the same concentration as the MAb, served as negative controls. The cells were washed three times in RBC lysing buffer (Sigma Chemical Co., St. Louis, MO), resuspended in PBS (pH 7.4), and then analyzed in FACScan (double labeling) using CellQuest software (Becton-Dickinson Immunocytometry, San Jose, CA).

Infection Assays

A pair of $CD4^+$ T cells at acute and convalescent phases were thawed, incubated in RPMI 1640 medium supplemented with 10% fetal bovine serum, and subjected to HIV-1 infection experiments without any stimulation. R5 HIV-1 AD8 and X4 HIV-1 NL4-3 stocks were propagated as described previously [Moriuchi et al., 1998a], tested for reverse-transcriptase (RT) activity, and used at 5,000 RT counts per 3×10^5 $CD4^+$ T cells. Viral replication was determined by RT assays, as described previously [Moriuchi et al., 1998a]. HIV-1 infection experiments were performed twice for each individual with similar results (data not shown).

RESULTS

As shown in Figure 1A, CCR5 expression was markedly increased on $CD4^+$ T cells in acute EBV infection as compared to that on control $CD4^+$ T cells. CCR5 expression levels were decreased to those comparable to control at convalescent phase. In contrast, CXCR4 expression was comparable between acute and convalescent phases (Fig. 1B).

Next, $CD4^+$ T cells obtained at acute or convalescent phases were infected with R5 or X4 HIV-1. $CD4^+$ T cells at acute phase of IM supported R5 HIV-1 infection more efficiently than did those at convalescent phase (Fig. 2A; Table I, patient 1). In contrast, $CD4^+$ T cells at neither acute nor convalescent phase efficiently supported X4 HIV-1 replication (Fig. 2B; Table I, patient 1), although these cells fully supported X4-HIV-1 infection upon

TABLE I. HIV-1 Replication in $CD4^+$ T Cells Derived From Patients With IM*

	Peak RT activity (cpm/μl)			
	R5-HIV-1		X4-HIV-1	
Patient	Acute	Convalescent	Acute	Convalescent
1	1721	501	298	252
2	1223	278	190	<100
3	2175	621	432	367
4	786	<100	<100	<100

*$CD4^+$ T cells were purified and frozen at acute and convalescent phases of IM. A pair of $CD4^+$ T cells at acute and convalescent phases were thawed and subjected to HIV-1 infection assays. Peak RT activities are shown.

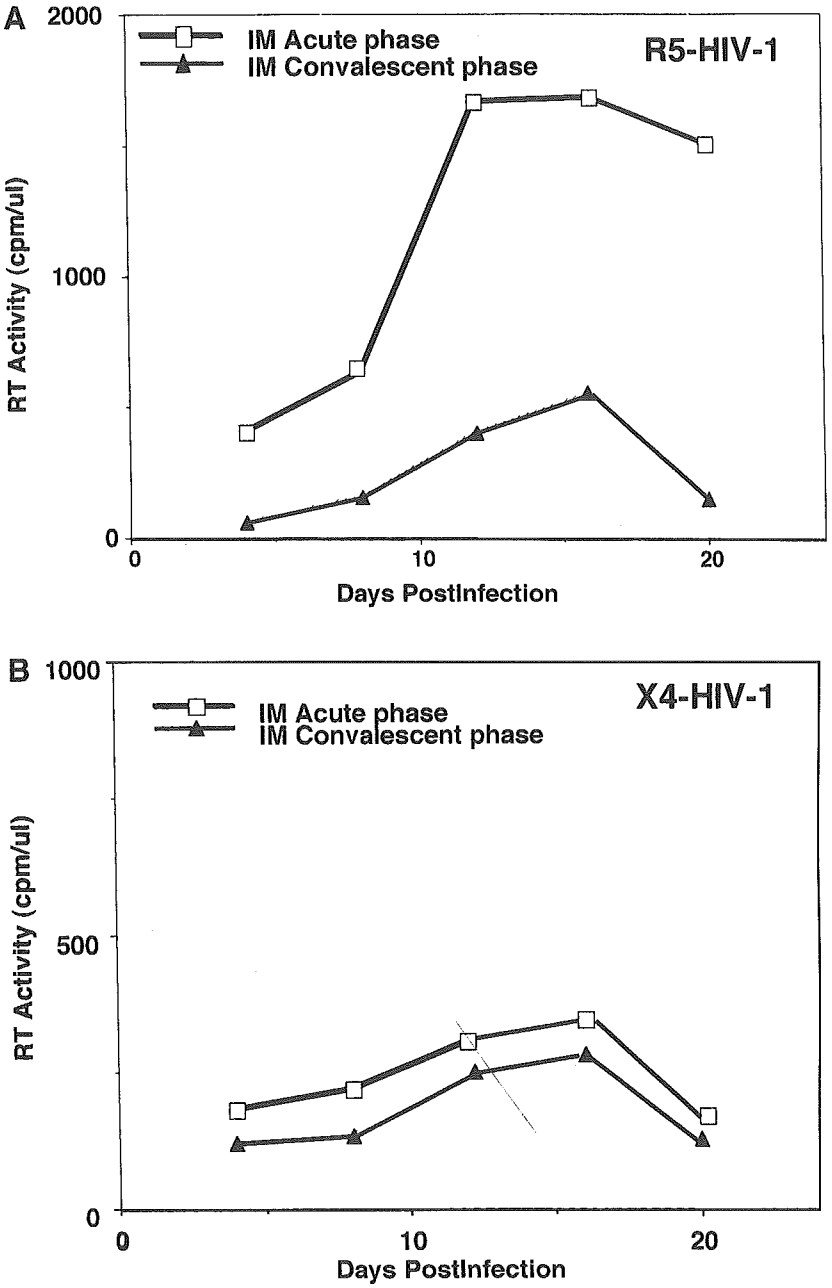

Fig. 2. Susceptibility of $CD4^+$ T cells derived from an EBV-infected individual to R5 (**A**) or X4 (**B**) HIV-1. Approximately 3×10^5 $CD4^+$ T cells derived from acute and convalescent phases of patient 1 were infected with HIV-1 AD8 (A) or HIV-1 NL4-3 (B), and cell-free supernatants were collected every four days for RT assays.

stimulation with phytohemagglutinin (data not shown). Similar results were obtained from three other IM patients examined (Table I, patients 2–4).

DISCUSSION

Our study has suggested that $CD4^+$ T cells derived from patients at acute phase of IM efficiently supported R5-HIV-1 infection without any artificial in vitro stimulation. An EBV-encoded transactivator, EB nuclear antigen-2, has been shown to upregulate HIV-1 long terminal repeat promoter in vitro [Scala et al., 1993]. However, such interaction requires coinfection of a single cell with both HIV-1 and EBV, which is unlikely based upon the fact that EBV DNA was not detected in $CD4^+$ T cells used for in vitro HIV-1 infection. A number of microbial infections induce lymphocyte activation, which has been associated with enhanced replication of HIV-1 in coinfected individuals [Blanchard et al., 1998; Moriuchi et al., 1998a,b, 2000a,b]. Although acute EBV infection also induced lymphocyte activation, it was not enough to support X4 HIV-1 infection. Therefore,

lymphocyte activation alone was not sufficient to increase susceptibility to R5 HIV-1. The precise mechanism whereby acute EBV infection favors replication of R5-HIV-1 remains obscure; however, increased CCR5 expression levels during acute EBV infection may also predispose efficient replication of R5 HIV-1.

Since EBV is transmitted by intimate contact such as kissing, it is not unreasonable to consider that both EBV and HIV can be transmitted simultaneously or sequentially. Furthermore, R5-HIV-1 is a transmitting virus, while X4-HIV-1 emerges upon disease progression [Zhu et al., 1993]. Therefore, possible interaction between R5-HIV-1 and EBV, as demonstrated in this study, may have implications on the pathogenesis of acute HIV infection. This hypothesis warrants further investigation.

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REFERENCES

Blanchard A, Montagnier L, Gougeon ML. 1998. Influence of microbial infections on the progression of HIV disease. Trends Microbiol 5:326–331.

Bonecchi R, Bianchi G, Bordignon PP, D'Ambrosio D, Lang R, Borsatti A, Sozzani S, Allavena P, Gray PA, Mantovani A, Sinigaglia F. 1998. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. J Exp Med 187:129–134.

Juffermans NP, Paxton WA, Dekkers PE, Verbon A, de Jonge E, Speelman P, van Deventer SJ, van der Poll T. 2000. Up-regulation of HIV coreceptors CXCR4 and CCR5 on $CD4^+$ T cells during human endotoxemia and after stimulation with (myco)bacterial antigens: the role of cytokines. Blood 96:2649–2654.

Moriuchi M, Moriuchi H. 2001. Octamer transcription factors upregulate the expression of CCR5, a co-receptor for HIV-1 entry. J Biol Chem 276:8639–8642.

Moriuchi M, Moriuchi H. 2003. YY1 transcription factor downregulates the expression of CCR5, a co-receptor for HIV-1 entry. J Biol Chem 278:13003–13007.

Moriuchi H, Moriuchi M, Fauci AS. 1997. Cloning and analysis of the promoter region of CCR5, a coreceptor for HIV-1 entry. J Immunol 159:5441–5449.

Moriuchi M, Moriuchi H, Turner W, Fauci AS. 1998a. Exposure to bacterial products renders macrophages highly susceptible to T-tropic human immunodeficiency virus type 1: implications for in vivo coinfections. J Clin Invest 102:1540–1550.

Moriuchi H, Moriuchi M, Fauci AS. 1998b. Factors secreted by HTLV-I infected cells can enhance or inhibit replication of HIV-1 in HTLV-I uninfected cells: implication for in vivo coinfection with HTLV-I and HIV-1. J Exp Med 187:1689–1698.

Moriuchi M, Moriuchi H, Fauci AS. 1999. GATA-1 transcription factor transactivates the promoter for CCR5, a co-receptor for HIV-1 entry. Blood 93:1433–1435.

Moriuchi H, Moriuchi M, Mizell SB, Ehler LA, Fauci AS. 2000a. In Vitro Reactivation of HIV-1 from Latently Infected, Resting $CD4^+$ T Cells upon Bacterial Stimulation. J Infect Dis 181:2041–2044.

Moriuchi M, Moriuchi H, Williams R, Straus SE. 2000b. Herpes Simplex Virus Infection Induces Replication of Human Immunodeficiency Virus Type 1. Virology 278:534–540.

Patterson BK, Czerniewski M, Andersson J, Sullivan Y, Su F, Jiyamapa D, Burki Z, Landay A. 1999. Regulation of CCR5 and CXCR4 expression by type 1 and type 2 cytokines: CCR5 expression is downregulated by IL-10 in CD4-positive lymphocytes. Clin Immunol 91:254–262.

Reynes J, Portales P, Segondy M, Baillat V, Andre P, Avinens O, Picot MC, Clot J, Eliaou JF, Corbeau P. 2001. CD4 T cell surface CCR5 density as a host factor in HIV-1 disease progression. AIDS 15:1627–1634.

Scala G, Quinto I, Ruocco MR, Mallardo M, Ambrosino C, Squitieri B, Tassone P, Venuta S. 1993. Epstein-Barr virus nuclear antigen 2 transactivates the long terminal repeat of human immunodeficiency virus type 1. J Virol 67:2853–2861.

Tkachuk AN, Moormann AM, Poore JA, Rochford RA, Chensue SW, Mwapasa V, Meshnick SR. 2001. Malaria enhances expression of CC chemokine receptor 5 on placental macrophages. J Infect Dis 183:967–972.

Wu L, LaRosa G, Kassam N, Gordon CJ, Heath H, Ruffing N, Chen H, Humblias J, Samson M, Parmentier M, Moore JP, Mackay CR. 1997. CCR5 levels and expression pattern correlate with infectability by macrophage-tropic HIV-1 in vitro. J Exp Med 185:1681–1691.

Zaunders JJ, Kaufmann GR, Cunningham PH, Smith D, Grey P, Suzuki K, Carr A, Goh LE, Cooper DA. 2001. Increased turnover of $CCR5^+$ and redistribution of $CCR5^-$ CD4 T lymphocytes during primary human immuodeficiency virus type 1 infection. J Infect Dis 183:736–743.

Zhu T, Mo H, Wang N, Nam DS, Cao Y, Koup RA, Ho DD. 1993. Genotypic and phenotypic characterization of HIV-1 patients with primary infection. Science 261:1179–1181.

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

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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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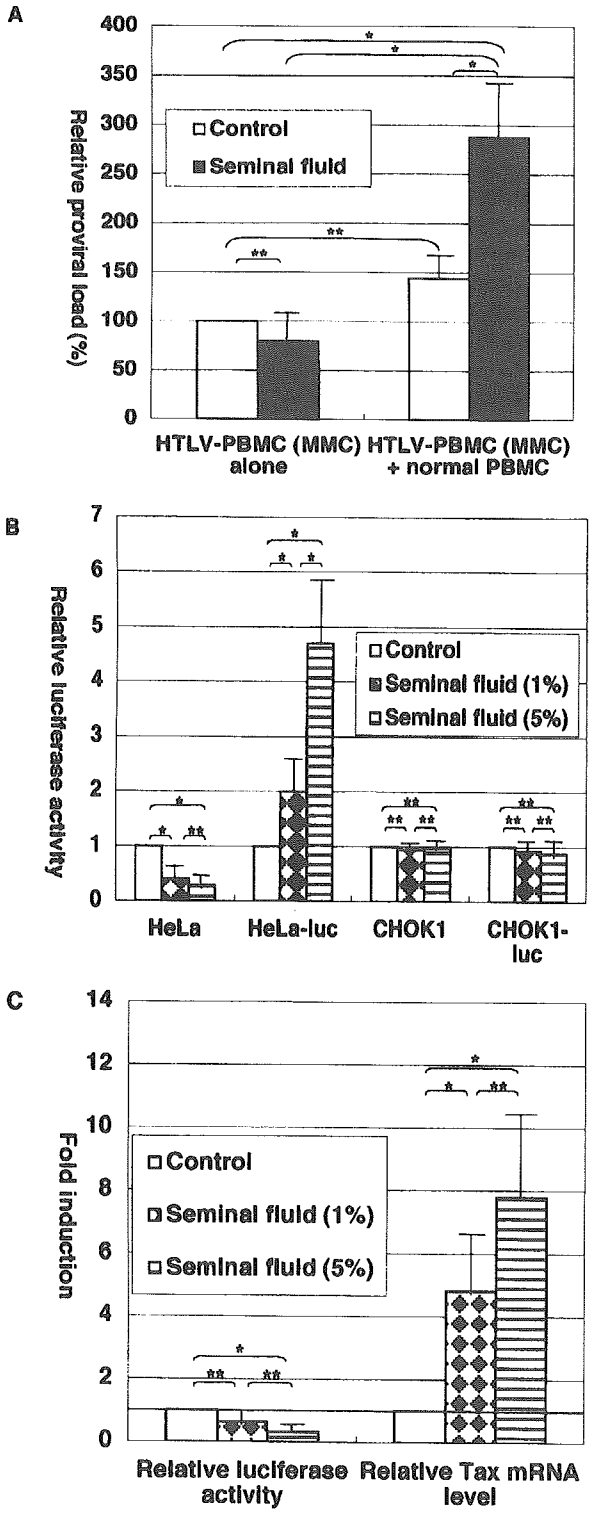

FIG. 1. Seminal fluid-mediated effects on HTLV-1 infection. (A) Seminal fluid facilitates de novo HTLV-1 infection. PBMC were isolated from three asymptomatic HTLV-1 carriers and treated with MMC as described previously (2). MMC-treated, HTLV-1-infected PBMC were cultured either alone or with PBMC derived 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.

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REFERENCES

1. **Anderson, D. J., J. A. Politch, L. D. Tucker, R. Fichorova, F. Haimovici, R. E. Tuomala, and K. H. Mayer.** 1998. Quantitation of mediators of inflammation and immunity in genital tract secretions and their relevance to HIV type 1 transmission. AIDS Res. Hum. Retrovir. **14**(Suppl. 1):S43–S49.
2. **Andrews, J. M., M. J. Oglesbee, A. V. Trevino, D. J. Guyot, G. C. Newbound, and M. D. Lairmore.** 1995. Enhanced human T-cell leukemia virus type I expression following induction of the cellular stress response. Virology **208**: 816–820.
3. **Benkirane, M., R. F. Chun, H. Xiao, V. V. Ogryzko, B. H. Howard, Y. Nakatani, and K.-T. Jeang.** 1998. Activation of integrated provirus requires histone acetyltransferase. P300 and P/CAF are coactivators for HIV-1 Tat. J. Biol. Chem. **273**:24898–24905.
4. **Busch, M. P., T. H. Lee, and J. Heitman.** 1992. Allogeneic leukocytes but not therapeutic blood elements induce reactivation and dissemination of latent human immunodeficiency virus type 1 infection: implications for transfusion support of infected individuals. Blood **80**:2128–2135.
5. **Jeremias, J. C., A. M. Bongiovanni, and S. S. Witkin.** 1999. Induction of heat shock protein expression in cervical epithelial cells by human semen. Infect. Dis. Obstet. Gynecol. **7**:17–22.
6. **Jeremias, J., and S. S. Witkin.** 1999. Effect of human seminal fluid on production of messenger ribonucleic acid for metalloproteinase 2 and metalloproteinase 9 in cervical epithelial carcinoma cells. Am. J. Obstet. Gynecol. **181**:591–595.
7. **Kibler, K. V., and K.-T. Jeang.** 2001. CREB/ATF-dependent repression of cyclin A by human T-cell leukemia virus type 1 Tax protein. J. Virol. **75**: 2161–2173.
8. **Miller, S. C., A. Taylor, K. Watanabe, K. Mok, and F. M. Torti.** 1997. Regulation of NF-κB and HIV-1 LTR activity in mouse L cells by ultraviolet radiation: LTR trans-activation in a nonirradiated genome in heterokaryons. Exp. Cell Res. **230**:9–21.
9. **Moriuchi, M., H. Moriuchi, S. E. Straus, and J. I. Cohen.** 1994. Varicella-zoster virus (VZV) virion-associated transactivator open reading frame 62 protein enhances the infectivity of VZV DNA. Virology **200**:297–300.
10. **Moriuchi, M., H. Inoue, and H. Moriuchi.** 2001. Reciprocal interactions between human T-lymphotropic virus type 1 and prostaglandins: implications for viral transmission. J. Virol. **75**:192–198.
11. **Moriuchi, M., and H. Moriuchi.** 2001. A milk protein lactoferrin enhances human T-cell leukemia virus type I and suppresses HIV-1 infection. J. Immunol. **166**:4231–4236.
12. **Moriuchi, M., and H. Moriuchi.** 2002. Transforming growth factor-β enhances human T-cell leukemia virus type I infection. J. Med. Virol. **71**:343–346.
13. **Moriuchi, M., and H. Moriuchi.** 2003. YY1 transcription factor downregulates the expression of CCR5, a co-receptor for HIV-1 entry. J. Biol. Chem. **278**:13003–13007.
14. **Okada, M., and K.-T. Jeang.** 2002. Differential requirements for activation of integrated and transiently transfected human T-cell leukemia virus type 1 long terminal repeat. J. Virol. **76**:12564–12573.
15. **Stuver, S. O., N. Tachibana, A. Okayama, S. Shioiri, Y. Tsunetoshi, K. Tsuda, and N. E. Mueller.** 1993. Heterosexual transmission of human T cell leukemia/lymphoma virus type 1 among married couples in southwestern Japan: an initial report from the Miyazaki cohort study. J. Infect. Dis. **167**:57–65.

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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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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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