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Doctor's Thesis

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Song Wei
宋 薇

指導教官
原田 信志 教授

熊本大学大学院医学研究科 感染防御学講座

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Wei Song

Department of Medical Virology, Kumamoto University School of Medicine, Kumamoto, Japan

March 2002

Supervised by

Professor Shinji Harada,
Chairman, Department of Medical Virology,
Kumamoto University School of Medicine,
Kumamoto, Japan

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Song　Wei

指導教官
熊本大学大学院医学研究科 感染防御学講座
原田　信志　教授

審査員名:　1）エイズ学研究センター
ウイルス制御分野担当教授　滝口　雅文　教授

2）エイズ学研究センター
病態制御分野担当教授　　松下　修三　教授

3）免疫識別学講座担当教授　　西村　泰治　教授

2002年3月

CONTENTS

SUMMARY

Capsianosides, diterpene glycosides, are extracted from *Capsicum* plants and have been shown to increase tight-junctional permeability of human intestinal Caco-2 cells by affecting the cytoskeletal function through modulating the reorganization of actin filaments. We have examined whether these compounds have any effect on human immunodeficiency virus type 1 (HIV-1) infection. The effect of *capsianosides* II, XI, A, and G on HIV-1 infection depended on the methods of treatment, cell lines and HIV-1 strains used. *Capsianoside* G had the strongest enhancement (8-fold) when MAGI/CCR5 cells were treated 1 hr before adding an X4 virus, whereas inhibition was observed when the cells were treated with the compound at the time of infection. Apparent enhancement of virus infection in MAGI/CCR5 cells was neither observed for the X4 virus by *capsianosides* II, XI, and A, nor for an R5 virus by *capsianoside* G. The enhancement of HIV-1 infection by *capsianoside* G was exclusively related to the usage of the CXCR4 coreceptor, and that was confirmed by an R5X4 virus using GHOST/CXCR4 cells. The *capsianoside* G-treated MAGI/CCR5 cells had no change in the expression level of CD4, CXCR4 and CCR5, however, colocalization and capping of CD4 and CXCR4, but not of CD4 and CCR5 was observed. Our results suggested that *capsianoside* G enhanced X4

virus infection at the levels of viral penetration through the capping and colocalization of receptors needed for infection.

要旨

植物（*Capsicum*）から抽出された糖脂質の一種であるカプシアノサイドは、細胞のアクチンフィラメントに作用することにより、腸上皮由来の細胞株である Caco-2 細胞の細胞間透過性を高めることが知られている。細胞膜に変化を与えると考えられるこの化合物(分子量と糖鎖の違いによりカプシアノサイド II、XI、A、G が存在する）がヒト免疫不全ウイルス 1 型（HIV-1）の感染にどのような影響を与えるか調べた。

HIV-1 としては、CD4 と CXCR4 とをレセプターとして使用する X4 株、CD4 と CCR5 とを利用する R5 株、CXCR4 と CCR5 の両方を用いる R5X4 株を使用した。感染には、MAGI/CCR5 細胞（CD4、CXCR4、CCR5 陽性で LTR/β-gal が組み込まれた HeLa 細胞）を用いた。感染価の評価には X-gal で核が青く染色される感染細胞を数えた。また、感染細胞培養上清中のウイルス蛋白 p24 も定量した。細胞表面の CD4、CXCR4、CCR5 発現は FACS や共焦点顕微鏡で観察した。

カプシアノサイド II、XI、A、G の効果は細胞への処理法、細胞の種類、使用したウイルス株間で異なっていた。HIV-1 X4 株を使用し、感染の 1 時間前に MAGI/CCR5 細胞をカプシアノサイド G で前処理すると、感染価は 8 倍上昇した。一方、感染と同時にこの化合物を細胞に作用させると、感染の抑制が認められた。カプシアノサイド G による HIV-1 感染の MAGI/CCR5 細胞における増強は、R5 ウイルスには認められなかった。更に、カプシアノサイド II、XI、A には HIV-1 感染促進効果は観察されなかった。CXCR4 と CCR5 の両方をレセプターとして使用する R5X4 ウイルス株では、カプシアノサイド G による感染の増強は CXCR4 を発現している細胞に特に強く認められた。従って、この物質による感染増強は CXCR4 特異的であると考えた。この特異性の理由を追究するため、HIV-1 のレセプター発現に変化があるか調べた。カプシアノサイド G 処理細胞では CD4、CXCR4、CCR5 の発現量に変化は見られなかったが、CD4 と CXCR4 のキャッピングと共集合が共焦点顕微鏡下で観察された。

化合物の中でも、2 量体で糖鎖の短いカプシアノサイド G が HIV-1 の X4 株の感染増強効果が最も大きかった。おそらく、このカプシアノサイド G は CD4 と CXCR4 とを架橋するようなかたちで反応するか、あるいは CD4 と CXCR4 とが細胞膜で集合するようなラフト形成を誘導するかで作用していると思われる。この細胞膜上での CD4 と CXCR4 の選択的集合が、結果として、HIV-1 の、特にこれらのレセプターを使用する X4 株の、選択的感染増強の原因となったと考えた。

PUBLICATION LIST

Main Publication

Authors

Wei Song, Shoji Yahara, Yosuke Maeda, Keisuke Yusa, Yuetsu Tanaka, and Shinji Harada

Title

Enhanced Infection of an X4 Strain of HIV-1 Due to Capping and Colocalization of CD4 and CXCR4 Induced by *Capsianoside* G, a Diterpene Glycoside

Journal

Biochemical and Biophysical Research Communications

Volume, Page, Year

283, 423-429, 2001

Others

1. Human T-cell Leukemia Virus Type-I (HTLV-I) Tax is not the Only One Factor to Enhance Human Immunodeficiency Virus Type-I (HIV-I) Infection in Culture-supernatants.
   Akira Tempaku, Yosuke Maeda, Wei Song & Shinji Harada
   Virus Genes 23: 77-80, 2001
2. Inhibitory Effect of Human Saliva on HIV-1 Infection.
   Etsuko Kumagai, Wei Song
   Japanese Journal of Clinical Immunology 24: 152-159, 2001

3. Construction of HIV-1 Library Containing Random Combinations of Amino Acid Substitutions in the HIV-1 Protease Due to Resistance by Protease Inhibitors.

Keisuke Yusa, Wei Song, Matthias Bartelmann & Shinji Harada

Journal of Virology: 76 (6) March, 2002 (in press).

ACKNOWLEDGMENTS

These research had been done during my five-years stay in Japan from 1997-2002, at the Department of Medical Virology, Kumamoto University School of Medicine.

It is my great pleasure to express my gratitude to Professor Shinji Harada, M.D. Chairman of the Department of Medical Virology. I would like to express my deepest sense of appreciation for giving me an excellent opportunity to study in his Lab and for his kind education, help, and continuous encouragement to make my research work successful.

I express my special regards and thanks to Dr. Yosuke Maeda, Associate Professor, Department of Medical Virology, for his valuable suggestion and technique education.

My great thanks are due to Dr. Keisuke Yusa, Department of Medical Virology, for leading me to the molecular virology field and kindly education.

My hearty thanks and gratitude to Mrs. Etsuko Kumagai, Department of Medical Technology, Kumamoto University College of Medical Science, for her kindness, friendly help me and my family during my studying in Kumamoto.

Thanks to all the members of the Lab for the technical cooperation in my research works.

My heartfelt thanks to Professor Akio Suzuki, Department of Biology, Faculcy of General Education Kumamoto University and his wife for their friendly help from the begining of my study in Japan. They supported my staying in Kumamoto like my parents.

My sincere thanks to Mr. T. Saijou, the president of Kyushu-fudosan company, for providing me financial support during my study so that I could concentrate on research. I also express my respect to him for his love for China and Chinese people, and has built a bridge of Japan-China friendship.

I give my grateful sense to my guarantee, Father Franco Sottocornola, and all members of the Center of Catholic of Live Mountain, Tamana, Japan, for their help and spirit encouragement to support my oversees life.

I express my regards and respects to my parents-in-law and my brother and sister-in law, who have been taking care of my daughter in Nanjing, China, and gave my daughter very patient and kind education during my study in Japan. Without their inspiration and support I would not have finished my study here. To my pretty daughter, she was just five

years old when I came to Japan, has been a source of my inspiration, I thank my daughter for her understanding and encouragement.

Lastly, I express my regards to my husband, Shu Zhao, who brought me to Japan and encouraged me to take the doctor course. He always listened and was a constant source of moral support. As well as my parents, aunt and uncle, whose continuous encouragement throughout my education, their dream gave me moral instinct to come to so far.

ABBREVIATIONS

AIDS:	acquired immune deficiency syndrome
CMV:	cytomegalovirus
DMEM:	Dulbecco minimal essential medium
ELISA:	enzyme-linked immunosorbent assay
FACS:	flourescence activated cell sorting
FBS:	fetal bovine serum
FITC:	fluorescein isothiocyanate
GFP:	green fluorescent protein
HIV-1:	human immunodeficiency virus type 1
HOS:	human osteosarcoma cell line
hr:	hour(s)
HSV:	herpes simplex virus
IgA:	immunoglobulin A
IgG:	immunoglobulin G
IgM:	immunoglobulin M
LTR:	long terminal repeat
mAb:	monoclonal antibody
MAGI:	multinuclear activation of a galactosidase indicator

min:	minute
NaN3:	sodium azide
O/N:	overnight
PBMC:	peripheral blood mononuclear cells
PBS:	phosphate-buffered saline
PHA:	phytohaemagglutinin
RT:	room temperature
TJ:	tight-junctional
TPA:	12-O-tetradecanoylphorbol-13-acetate
VZV:	varicella-zoster virus
X-Gal:	5-bromo-4-cholro-3-insilyl-b-D galactopyranoside

BACKGROUND

1. BACKGROUND

1.1. Introduction

Over the 20 years since it was first identified, the HIV/AIDS epidemic has threatened the world health. An estimated 36.1 million people worldwide are currently living with HIV or AIDS and 21.8 million people have already died since the beginning of the epidemic; and 13.2 million children have become "AIDS orphans", having lost their mother or both parents to the disease (1). More than 14,000 new infections occur daily, namely 5.3 million in 2000 alone, while 3.0 million people died from AIDS (1). The HIV/AIDS epidemics continue to spread unequally around the world. Approximately 70 percent of cases occur in sub-Saharan Africa, where, in some regions, the seroprevalence of HIV among adults exceeds 25 percent. The Caribbean, Southeast Asia, and eastern Europe are also struggling with substantial rates of new infection. East Asia and Pacific are still keeping HIV at bay, in regions that include some of the world's most populous countries. An estimated 640,000 people were living with HIV or AIDS at the end of 2000 in this area. This represents just 0.07 percent of the regions' adult population, as compared with the prevalence rate of 0.56 percent in south and southeast Asia. However, their is undeniable potential for increasing spread in east Asia.

The HIV epidemic is linked closely to an epidemic of injecting drug use in many countries of the region (2). In China, sentinel surveilance

among injecting drug users detected no HIV infection in any of eight sentinel sites in 1995, but HIV was found in 17 of 19 sites in 1998. The highest prevalence among injecting drug users was 82 percent in Yining city in the western Xinjiang province (3). Sentinel surveillance among sex workers in China shows little or sporadic condom use, and HIV prevalence ranging up to 6% in 1999 (4). Having practically eradicated sexually transmitted infections by the 1970s, China is now seeing a steep rise in the number of reported cases: from 5,800 in 1985 to over 236,000 in 1999 (5). This increase in sexually transmitted infections indicates potential for HIV spread in the future. Because surveillance efforts in China have focused primarily on relatively small populations of high-risk groups, the true scope of China's HIV/AIDS epidemic is unknown. But it is clear the situation is serious. Chinese officials estimate that 600,000 Chinese are now infected with HIV. But international groups say the true number is closer to 1.5 million and predict that if the epidemic continues to spread at its current rate that number will climb to 10 million over the next four years and to 20 million by the end of 2010 (6).

Since the discovery of the first HIV isolate, the morphological, physical, structural and functional features of HIV isolates have been extensively studied and reviewed (7~10). The mechanism of HIV, a member of the retrovirus family *Retroviridae,* infection (11~13) has been deeply studied over two decades. Like all other viruses, HIV is an

intracellular parasite and its life cycle includes two distinct stages: an early phase and a late phase, each consists of sequential steps, and several of these steps involve specific interaction of viral protein and nucleic acids with host cell factors (14). The early phase begins with attachment of a virion to a cell surface receptor and continues to formation of a provirus integrated into the host cell genome (14). The process of viral entry is a key step in the initiation of HIV-1 infection. HIV-1 enters permissive cells by binding to cellular receptor on the target cell surface, CD4, followed by gp120-gp41-mediated fusion of the viral and target cell membranes (15~17). CD4 has long been known as an essential receptor for HIV-1 entry, however it is not sufficient to confer transmission of virus to most nonhuman and some human cell lines that are resistant to HIV-1 infection (18, 19). Several chemokine receptors, members of a family of seven-transmembrane G protein-coupled chemokine receptors (20~22) have been shown to function as coreceptors (15, 17, 23, 24). Most primary isolates use CD4 together with the CCR5 chemokine receptor (R5 strains), while some primary syncytium-inducing as well as T-cell line-adapted strains use the CXCR4 coreceptor (X4 strains), dualtropic strains utilize both CXCR4 and CCR5 coreceptors (R5X4 strains) (25). HIV-1 entry consists of distinct sequential steps such as attachment of the virus to a specific cell surface receptor, fusion between the viral envelope and the cell membrane, and penetration of the viral genome into target cells. These process of virus infection could be affected by various external and internal factors (26~30)

resulting in the enhancement or the inhibition of HIV-1 replication. Numerous in vitro studies have revealed that some compounds like glycosaminoglycans and glycolipids apparently modulate HIV-1 infection and fusion (31, 32). After receptor binding, structural rearrangements of gp41 subunit are thought to play a role in membrane fusion (33~35) in a process which has often been compared to that used by the hemagglutinin HA2 protein of influenza virus (36~38). Based on analogy of similar rearrangements in the glycoproteins of influenza virus (39) and Rous sarcoma virus (40), the CD4-induced conformational change in the HIV-1 glycoprotein is believed ultimately to lead to the insertion of the fusion peptide into the target cell membrane (33). The V3 loop of HIV-1 gp 120 can bind to glycosphingolipids (galactosylceramide and ganglioside GM3) (41, 42) which are expressed on human $CD4^+$ lymphocytes (43). Moreover, $CD4^+$ cells are rendered competent to CD4-dependent CXCR4-utilizing HIV-1 fusion by transfer of human erythrocyte glycosphingolipids (globotriaosylceramide) (44). Globotriaosylceramide (Gb3) in $CD4^+/CXCR4^+$ cells but not $CD4^+/CXCR4^-$ cells allows fusion with HIV-1LAI-envelope glycoprotein expressing cells (TF228). Therefore, Gb3 functions in conjunction with HIV-1 coreceptor, CXCR4 to promote fusion proposing the Gb3 functions by recruiting CD4 and /or CXCR4 at the fusion site through structurally specific interactions (45). These data support the concept that some glycolipids may function as alternative

fusion cofactors or crosslinker between CD4 and coreceptors and affect the strucure of host cells consequently modify the degree of virus infection.

It was reported that *capsianosides* extracted from *Capsicum* plants affected the cytoskeletal elements, located underneath the cell surface, by modulating the reorganization of actin filaments, and thus changing the tight-junctional structure and permeability (46). It was also suggested that these cytoskeletal elements may play an important role in the distribution of distinct membrane molecules in epithelial cells. Based on these premises, we aimed to elucidate the effect of *capsianosides* on HIV-1 infection. Most striking was the observation that *capsianoside* G selectively enhanced X4 virus infection when MAGI/CCR5 cells were pretreated with the compound. Moreover, *capsianoside* G induced capping and colocalization of CD4 and CXCR4 on target cells, and therefore, only enhancing the infection of X4 strains of HIV-1. Increased membrane fluidity is also suggested to promote the penetration of the virus.

1.2. Structure of *Capsianosides*

Capsicum, red pepper in solanaceous plants is a very important spice. With regard to the constituents of red pepper, the less polar ones have previously been extensively studied, however, the polar ones have not been sufficiently examined (47). As to the water-soluble constituents in *Capsicum* plants, only a furostanol glycoside, *capsicoside*, from seeds and roots of C. *annuum* was known (48) . The water-soluble constituents of

Capsicum fruits have been isolated, in which, *Capsianosides* II and XI were identified as monomeric compounds of acyclic diterpene glycoside, and *Capsianosides* A and G as esters of acyclic diterpene glicoside. Both *Capsianoside* II and *Capsianoside* A have a longer sugar chain comparing with *Capsianoside* XI and G (Fig. 1).

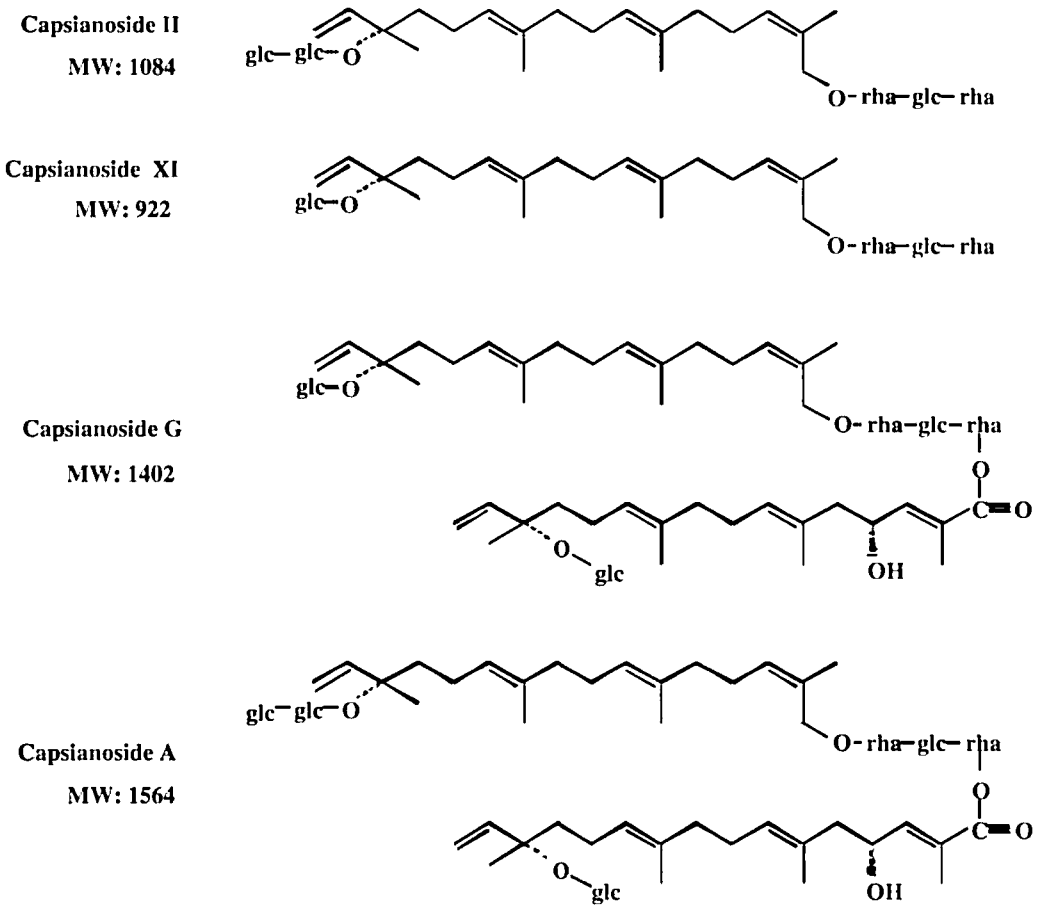

FIG. 1. Structure of *capsianosides*. MW: molecular weight.

1.3. Known Function of *Capsianosides*

Previous study (49) revealed that a sweet pepper extract enhanced the tight-junctional (TJ) permeability of a human Caco-2 cell monolayer. Another research by the same group further clarified that the active substance which modulated the TJ permeability were *capsianoside* A-F, diterpene glycosides. Treatment of the cells with *Capsianoside* F, the most active compound, decreased the cellular G-actin content by 40% and increased the F-actin content by 16%. The effect of *capsianoside* F was significantly suppressed by disturbing the cytoskeletal structure with cytochalasin D at a low dose (50ng/ml), suggesting that *capsianosides* affected the cytoskeletal function by modulating the reorganization of actin filaments, by which the TJ structure and permeability were changed (46). *Capsianoside* G is a later isolated analogue glycoside of *capsianoside* F, has a similar characteristics to *capsianoside* F.

1.4. Purpose of the Present Study

According to previous research, we found the different effect of glycolipid on HIV-1 infection related to treating condition and glycolipid itself (Table 1).

Table 1. Effect of glycolipids on HIV infection

Lipids	1hr before infection	same time	1hr after infection
Sphingomyelin	ND	no effect	weak enhance
Sphingosine	ND	no effect	no effect
Sphingosylphorylcholine	ND	no effect	ND
Lysophosphatidic acid	no effect	no effect	ND
Psychosine	ND	no effect	ND
Galactosylceramide	ND	inhibition	ND
Monosialoganglioside GM1	ND	inhibition	ND
Capsianoside II	weak enhance	inhibition	weak enhance
Capsianoside XI	weak enhance	inhibition	weak enhance
Capsianoside G	enhance	inhibition	inhibition
Capsianoside A	enhance	inhibition	inhibition
Fattiviracin A1	enhance	inhibition	inhibition

(ND: not determind)

Some enhancement was observed when the target cells were treated with glycolipids before infection, while inhibition in treating cells during HIV-1 infection. For instance, Fattiviracin A1, a neutral glycolipid, isolated from *Streptomyces* shows potent antiviral activites against herpes simplex virus (HSV), Varicella-zoster virus (VZV) and influenza A virus as well as HIV-1 (29, 50, 51). The initail aim of the present study was to define the suppressive mechanism of *capsianosides* on HIV-1 infection. However, we observed the dichotomous effects (inhibition and stimulation) of the *capsianosides* on HIV-1 infection, especially, *capsianoside* G showed 8-fold higher enhancement when the target cells were treated before HIV-1 infection. We focused on this stimulation phenominon to investigate the mechanism of *capsianoside* G on HIV-1 adsorption and infection in vitro.

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. Compounds

Capsianosides were extracted from *Capsicum* annuum and their structures were elucidated by spectroscopic and chemical means (47, 52). All *capsianosides* were dissolved in methanol at 10 mg/ml concentration and stored at –20°C until use. The volume of *capsianosides* required for experiments was dried in an eppendorf tube using a vacuum centrifugal concentrator (Tomy, CC-100), then dissolved in the appropriate medium for each experiment. No cytotoxicity of *capsianosides* was observed by sulfonated tetrazolium salt, 4[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate (WST-1) assay (53, 54), when the cells were treated with less than 400 µg/ml of each *capsianoside*.

2.2. Cells

MAGI/CCR5 cells were derived from HeLa-CD4/LTR-ß-Gal cells, which were transfected with pZeoSV-CCR5 using the calcium phosphate method as previously described (55). MAGI/CCR5 cells were maintained in Dulbecco minimal essential medium (DMEM) (ICN, Costa Meda, CA) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco), 2 mM L-glutamin, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 0.1 mg/ml G418, 0.05 mg/ml hygromycin B and 0.05 mg/ml of zeomycin.

MOLT4 cells were cultured in RPMI-1640 medium (Gibco) supplemented with 100 IU/ml penicillin, 0.1 mg/ml streptomycin and 10% heat inactivated FBS. GHOST cells, derived from a human osteosarcoma cell line (HOS) (provided by Dr. Vineet N. Kewalramani and Dr. Dan R. Littman through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) were maintained in DMEM supplemented with 10% FBS and antibiotics as indicated (56). These cells were stably transfected with human CD4 (GHOST/P), green fluorescent protein (GFP) reporter gene under control of the HIV-2 long terminal repeat, and one of the secondary chemokine receptors CXCR4 (GHOST/CXCR4) and CCR5 (GHOST/Hi5).

2.3. Preparation of virus stocks

The KMT strain, a dualtropic HIV-1 virus, was isolated from an HIV-1 infected patient, with AIDS stage A3, according to the Centers for Disease Control (CDC, Atlanta, GA) classfication of disease stage, and a CD4 T cell count of 7 cells/mm^3 (57). HIV-1 clone number 2 (HIV-1-C-2), a T-tropic strain, was originally obtained by the plaque cloning method (58) from the IIIB (LAI) isolate. HIV-1-C-2 (LAI) and KMT viruses were propagated in persistently infected producer MOLT4 cells (59) and CEM cells (57) respectively. Cell-free viruses were obtained by filtration of the supernatants of persistently infected cells through 0.45 μm-pore-size filters (Millipore, Bedford).

JR-FL, an M-tropic strain, was recovered by the transfection of pJR-FL into COS-7 cells using Lipofectamine (Gibco) (55). The p24 antigen of LAI, JR-FL and KMT virus stocks used in experiments was quantified by the p24 antigen enzyme-linked immunosorbent assay (ELISA) (Abbott) according to the manufacturer's protocol. Viruses were aliquoted and frozen at –80°C.

Env-pseudotyped viruses with the luciferase reporter gene were produced by the calcium phosphate method (24, 60~62). The 293T cells were cotransfected with the Env-deficient NL4-3 construct carrying the luciferase reporter gene pNL-Luc and with a pCXN vector expressing one of the envelope glycoproteins derived from NL4-3, KMT or JR-FL.

2.4. Multinuclear activation of a galactosidase indicator (MAGI) assay

MAGI assay is a method of titrating HIV-1 based on activation of an integrated LTR-ß-galactosidase gene in a $CD4^+$ cell, exploits the ability of the viral Tat protein to transactive an integrated ß-galactosidase gene driven by the HIV-1 long terminal repeat (LTR) promoter in $CD4^+$ cells (here we used MAGI/CCR5 cells, CD4-LTR/ß-gal HeLa cells expression CCR5) (Fig. 2). Individual infected cells or syncytia are counted in situ with a light microscope by virtue of their blue color (Fig. 3) that results from the hydrolysis and staining of the substrate 5-bromo-4-cholro-3-insilyl-ß-D galactopyranoside (X-Gal) by ß-galactosidase. To improve the

visualization of infected cells, the ß-galactosidase gene has been modified to include the nuclear localization signal of the SV 40 T antigen, thereby concentrating the blue color into the nucleus. We did our experiment by following the protocol as Fig. 4.

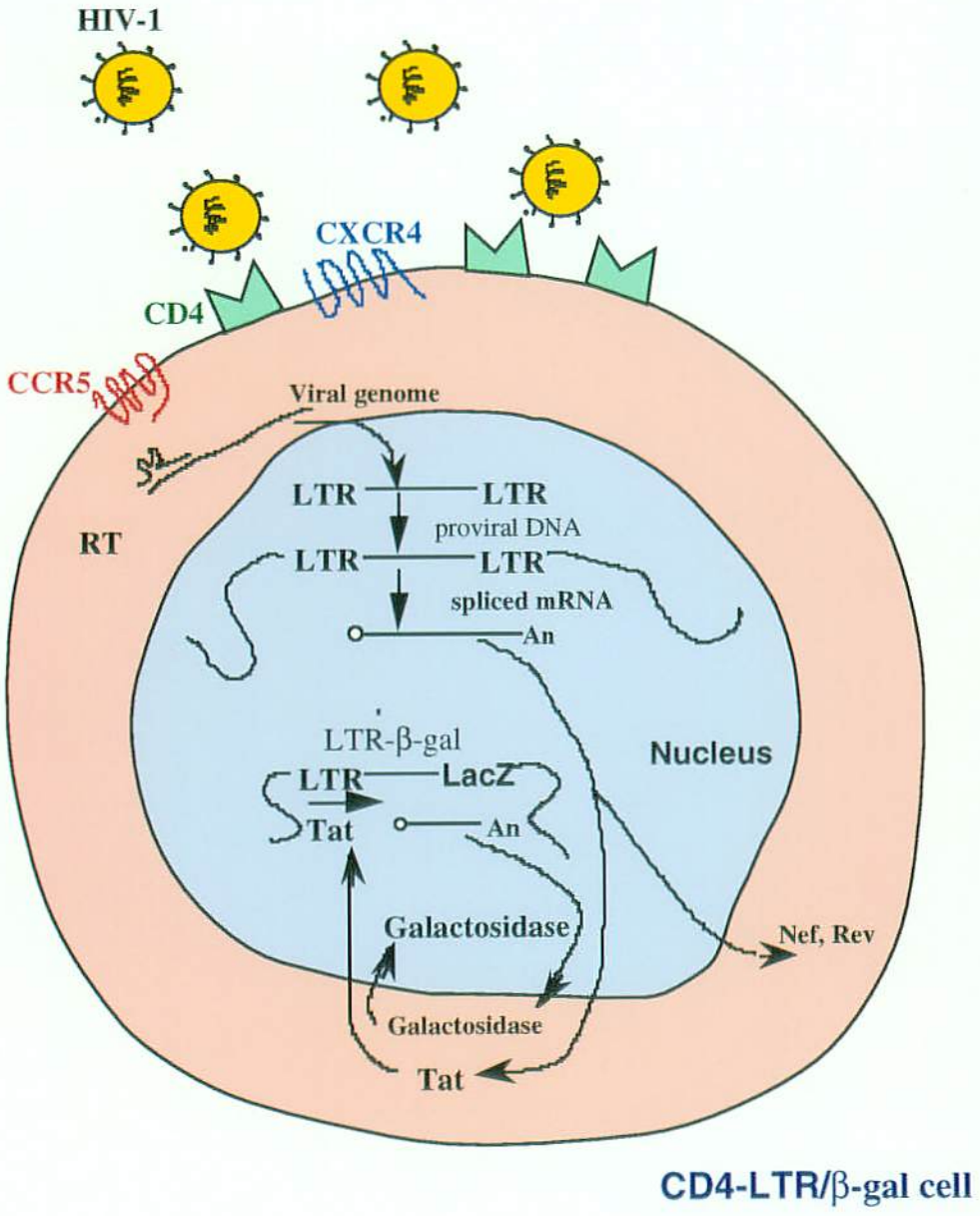

Fig. 2. Multinuclear Activation of a Galactosidase Indicator Assay (MAGI Assay)

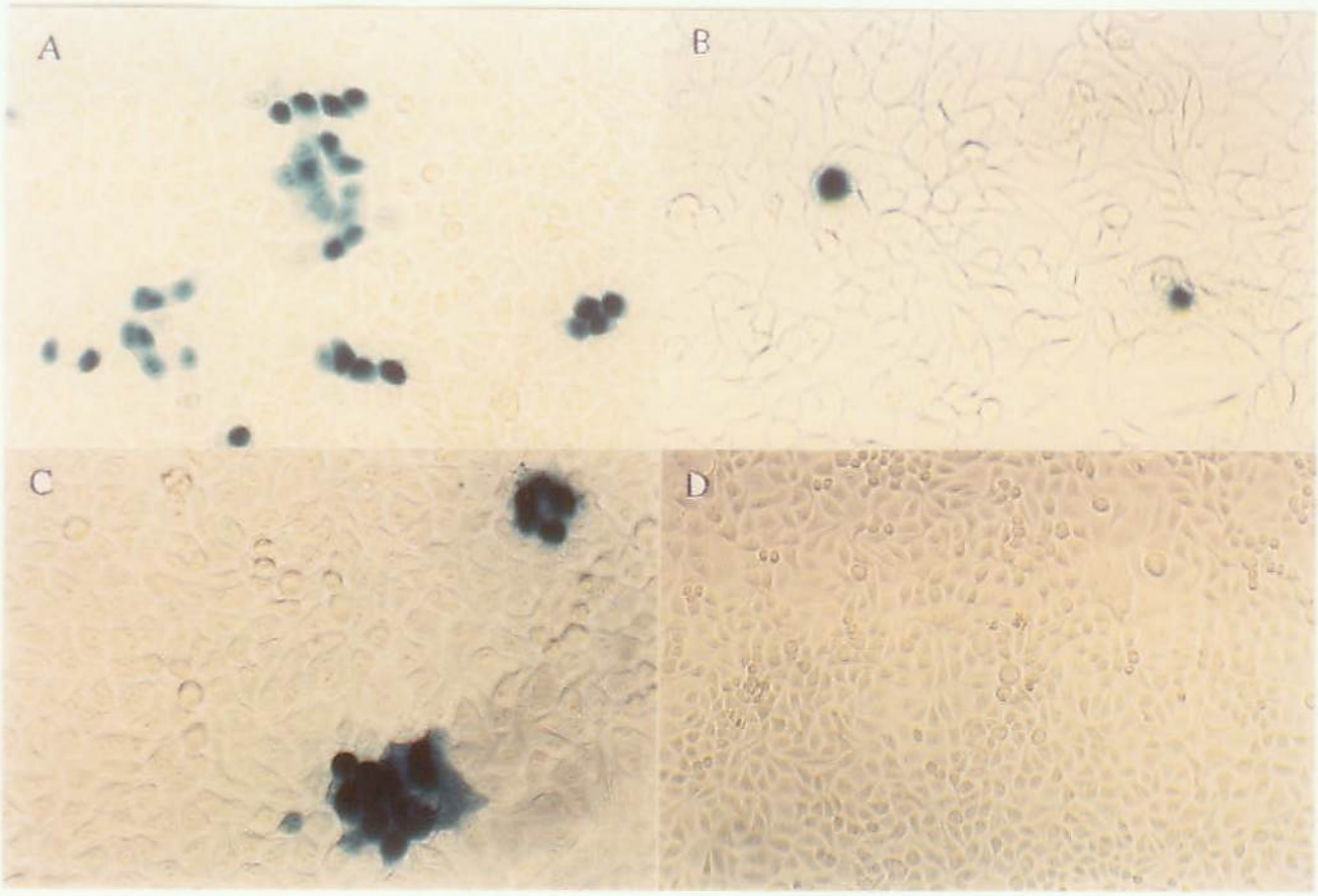

Fig. 3. The MAGI assay. MAGI/CCR5 cells were fixed and stained as described in text. Magnification x 20. (A) Infection of MAGI/CCR5 cells with a high titer HIV-1LAI virus stock, many cell nuclei are darkly stained. These nuclei are dark blue. (B) Infection of MAGI/CCR5 cells at a low dilution of virus. (C) Syncytium of infected MAGI/CCR5 cells. (D) Mock infection of the MAGI/CCR5 cells (Magnificcation x 10).

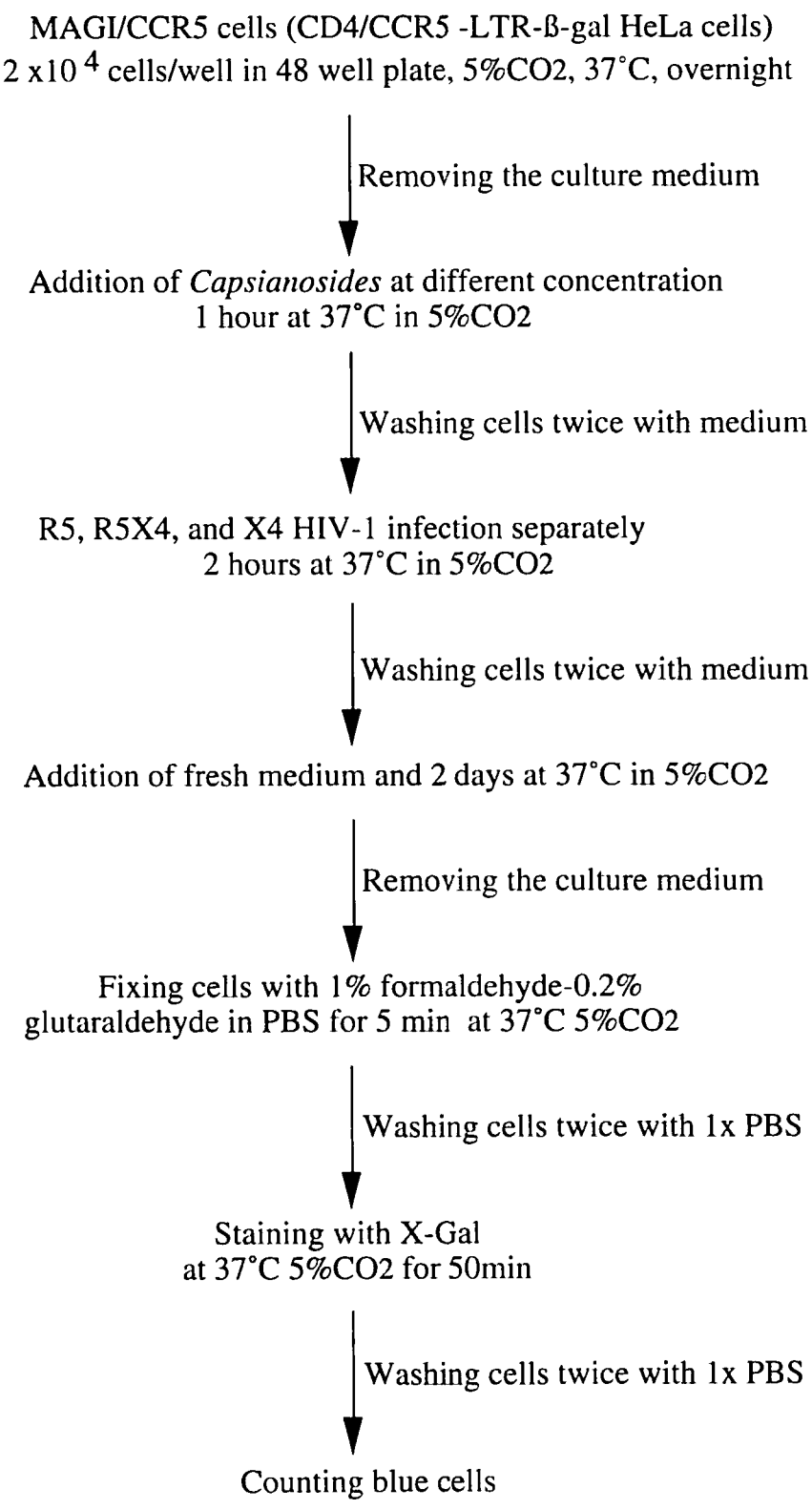

Fig. 4. MAGI assay for *capsianosides* activity on HIV-1 infection

The MAGI/CCR5 cells were seeded in 48-flat well plates (Iwaki, Tokyo, Japan) at 2 x 10^4 cells per well. On the following day, cells were treated with various concentrations (3.13 μg/ml to 400 μg/ml) of *capsianosides* for 1 hr at 37°C. Cells were washed with DMEM and inoculated with equal amounts (1.5 ng) of either LAI, JR-FL or KMT viruses. On day 2 postinfection, the medium was removed and the cell monolayer was fixed at 37°C with 200 μl per well of 1% formaldehyde-0.2% glutaraldehyde in phosphate-buffered saline (PBS) for 5 min. Cells were then washed twice with PBS and incubated for 50 min at 37°C with 200 μl per well of 4 mM potassium ferrocyanide, 2mM MgCl2, and 0.4 mg of X-Gal. The reaction was stopped by removing the staining solution and washing the cells twice with PBS. Blue cells were counted under microscope (63).

2.5. Infectivity assay with GFP readout

GHOST cells were seeded in 24-flat well plates (Iwaki, Tokyo, Japan) at 1.5 x 10^4 cells per well. On the following day, cells were treated with 200 μg/ml or 400 μg/ml of *capsianoside* G at 37°C for 1 hr. After washing once with DMEM, GHOST/CXCR4 and GHOST/Hi5 cells were infected with 4.5 ng KMT virus. On day 2 postinfection, cells were harvested, washed once with PBS, and fixed with 4% paraformaldehyde at 4°C and light-protected for 1 hr. Infected cells were then analyzed by

FACScan flow cytometer (Becton Dickinson, San Jose, CA) for GFP expression.

2.6. Infectivity assay with luciferase readout

MAGI/CCR5 and GHOST cells were seeded one day before infection in 48-flat well plates at 2 x 10^4 and 1.5 x 10^4 cells per well, respectively. Cells were treated with *capsianoside* G at 37°C for 1 hr and then infected with 2 ng per well env-pseudotyped luciferase reporter viruses. Two days after infection, cells were lysed with 100 µl per well of luciferase assay buffer (Promega). Luciferase activity was measured by adding 50 µl of the luciferase assay substrate (Promega) to 10 µl of cell lysate and reading the light activity in a luminometer detector (Lumat LB 9501/16; EG & G Berthold, Bad Wildbad, Germany).

2.7. Flow cytometric analysis for receptor and coreceptor expression level

Adherent MAGI/CCR5 and GHOST cells were detached from culture dishes by the use of a cell dissociation solution (Sigma, St, Louis, Mo., USA) (64). The monoclonal antibodies (mAb) used were Leu3a (anti-CD4), 2D7 (anti-CCR5) and 12G5 (anti-CXCR4, Pharmingen). Fluorescein isothiocyanate (FITC)-conjugated goat affinity purified antibody to mouse immunoglobulins (IgG, IgA, IgM) (Organon Teknika Corp., West Chester) was used as a secondary antibody. Flow

cytometric analysis was performed on a FACScan analyzer (Becton Dickinson).

2.8. Virus binding assay

The *Capsianoside* G influence on virus binding was measured p24 antigen of bound virus lysates on the target cells. The method is as below.

1. Seed 4 x 10^4 MAGI/CCR5 cells on 48-well plate 37°C O/N

2. Remove culture medium from the plate

3. Addition of serially diluted *capsianoside* G in

   DMEM in tripulicate wells 37°C 1 hr

4. Wash cells with 10%FBS-DMEM Once

5. Addition of HIV-1LAI (3 ng/well) in 100 ml

   DMEM 37°C 1 hr

6. Wash cells with 10%FBS-DMEM Twice

7. Wash cells with 1 x PBS Once

8. Addition of 1% tryton-X 100 (200ml/well) 37°C 1 hr

9. Plate shaker RT 20 min

10. Transfer lysates to eppendorf tube

11. Assess the amount of HIV-1 p24 antigen by ELISA (Abbott).

2.9. Immunofluorescent staining of viral receptor and coreceptors

Localization of receptor and coreceptor of MAGI/CCR5 cells was detected by Immunofluorescent staining of viral receptor and coreceptors following the protocol as below.

1. Confluently grown MAGI/CCR5 cells in glass-bottom

   dishes (10-mm diameter) (Matsunami, Japan) 37°C O/N

2. Remove culture medium from the dishes

3. 400 μg/ml of *capsianoside* G in DMEM medium

   or DMEM for negative control 37°C 1 hr

4. Remove *capsianoside* G and medium

5. Wash cells with 1% FBS-0.02% NaN_3-1xPBS Once

6. Fix cells with 4% paraformaldehyde (150ml/well) RT 15 min

7. Wash with 1% FCS-0.02% NaN_3-1xPBS Twice

8. Stain cells with 100 ml/well anti-CXCR4 (A145) or

   anti-CCR5 (T227) mAb separately for both

capsianoside G treated or control dishes. A145 and T227 mAbs recognized N-terminal portion of CXCR4 and CCR5 receptors, respectively. The mAbs were 1:100 diluted in 1x PBS. On ice 30 min

9. Wash cells with 1% FBS-0.02% NaN_3-1xPBS Twice

10. Stain cells with rhodamine conjugated anti-rat IgG (100 ml/well) On ice 30 min

11. Wash cells with 1% FBS-0.02% NaN_3-1xPBS Twice

12. Stain cells with 1:10 diluted FITC-labelled anti-CD4 mAb (Ortho Diagnostics, Raritin, N.Y.,USA) On ice 30 min

13. Wash cells with 1% FBS-0.02% NaN_3-1xPBS Twice

14. The glass-bottom dishes were mounted with immunofluorescence mounting solution (Molecular Probes, Engene, Oregon USA)

15. Apply laser scanning confocal microscope (LSM510 Zeiss equipped with Plan-Neofluar 100 x oil immersion objective and an Ar488nm/He-Ne543 nm laser).

RESULTS

3. RESULTS

3.1. Different effects of *capsianosides* on HIV-1 infection

The effect of *Capsianosides* II, XI, A, and G on HIV-1 infection was examined by MAGI assay with LAI (X4) strain of HIV-1 2 days post infection. The MAGI/CCR5 cells were treated with serial concentration of *capsianosides* before, during or after HIV-1LAI infection separately and assayed on day 2 after infection. The process was as follows (Fig. 5).

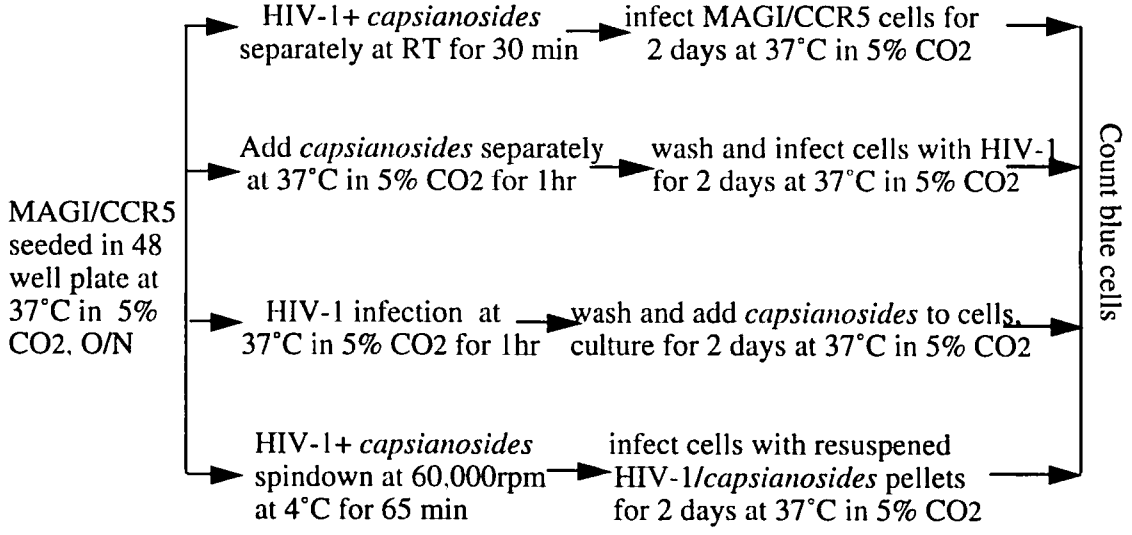

Fig. 5. Screening methods for effect of *capsianosides* on HIV-1 infection

The *capsianosides* showed the different effects on X4 HIV-1LAI infection depending on the compounds-treatment methods of target cells with HIV-1. The esters of acyclic diterpene glycoside, *capsianosides* G

and A inhibited HIV-1 virus infection except pretreating the cells with them before virus exposure, whereas the monomeric compounds of acyclic diterpene glycoside, *capsianosides* II and XI enhanced HIV-1 virus infection except compounds mixed with virus for 30 min before adding to MAGI/CCR5 cells (Table. 2). The most active substance is *capsianoside* G, which increased HIV-1_{LAI} infection of MAGI/CCR5 cells by 8-fold, therefore we focused on *capsianoside* G for the further research.

Table. 2 The Different Effects of *Capsianosides* (400μg/ml) on HIV-1_{LAI} Infection of MAGI/CCR5 Cells

Compounds	Compound+virus[1]	Compound first[2]	Virus first[3]	Compound+virus/spindown[4]
Capsianoside G	Block(0.91)	Enhance(8.19)	Block(0.69)	Block(0.99)
Capsianoside A	Block((0.57)	Enhance(2.04)	Block(0.54)	Block(0.88)
Capsianoside XI	Block(0.22)	Enhance(1.50)	Enhance(1.50)	Enhance(2.45)
Capsianoside II	Block(0.39)	Enhance(1.43)	Enhance(1.43)	Enhance(1.29)

[1]Compounds+virus at RT for 30 min before adding to MAGI/CCR5 cells.

[2] MAGI/CCR5 treated with compounds for 1hr at 37°C before HIV-1LAI infection.

[3] Adding compounds to MAGI/CCR5 cells after 1hr of HIV-1LAI infection.

[4] Centrifuge compounds and HIV-1LAI mixture at 4°C at 60,000 rpm for 65 min. Pellets were resuspended and used to infect MAGI/CCR5 cells.

3.2. Enhancement of X4 HIV-1 infection by *capsianoside* G in MAGI/CCR5 cells

Focusing on the effect of *capsianosides* on HIV-1 infection of pretreated MAGI/CCR5 cells, we found *capsianoside* G, which had a high molecular weight (1402) with short sugar chains, had the strongest enhancing effect on HIV-1 infection when the cells were treated for 1 hr at 37°C before adding the X4 virus (Fig. 6). Although 400 μg/ml of *capsianoside* G showed an 8-fold enhancement in a dose-dependent manner, we found no obvious enhancement by *capsianosides* II, XI, and A with X4 virus (Fig. 6). For some reason, the infection was inhibited by 91% when the MAGI/CCR5 cells were treated with 400 μg/ml of *capsianoside* G and X4 virus at the same time and during cultivation (Table. 2).

In order to further investigate whether *capsianoside* G acted in a viral type-specific manner, we used three different HIV-1 strains [LAI (X4), JR-FL (R5) and KMT (R5X4)] to infect compound-treated MAGI/CCR5 cells. Fig. 7A shows that the level of enhanced HIV-1 infectivity was much higher in LAI virus infection rather than that in KMT and JR-FL virus infections by *capsianoside* G. Similar findings were obtained by using env-pseudotyped luciferase reporter viruses (Fig. 7B). When the cells were treated with *capsianoside* G at 4°C for 1 hr, HIV-1 infection was unaffected (data not shown). Thus, the *capsianoside* G

enhanced HIV-1 infection in MAGI/CCR5 cells was exclusively related only to the X4 virus.

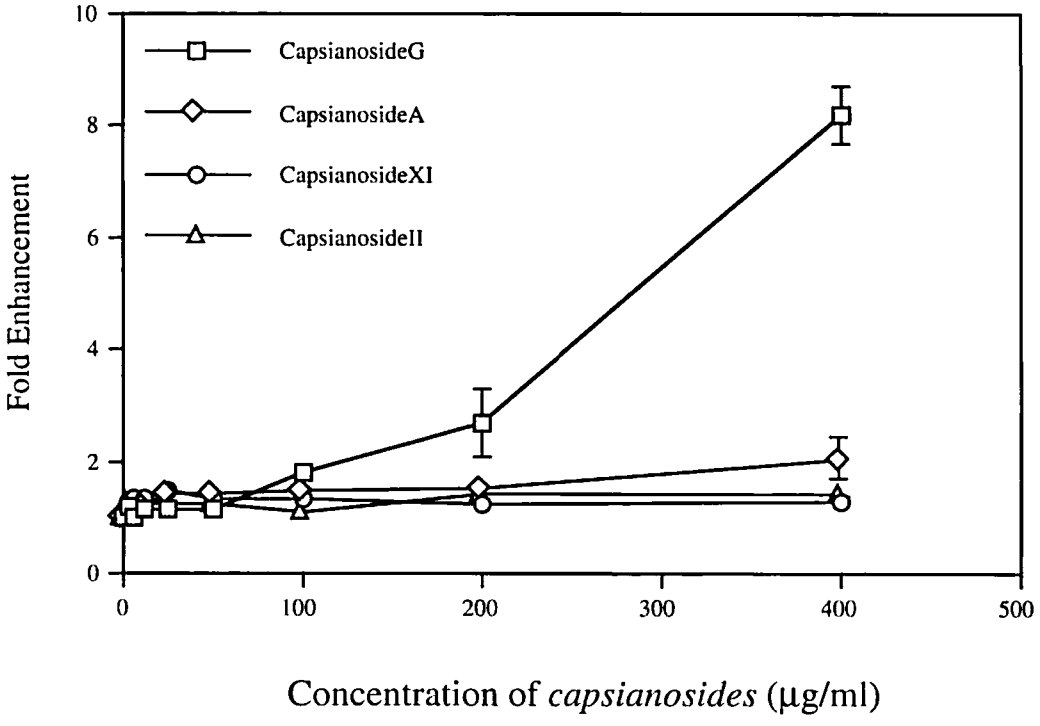

FIG. 6. Effect of *capsianosides* II, XI, A and G on HIV-1LAI (X4) infection of MAGI/CCR5 cells. The cells were treated with *capsianosides* at different concentrations for 1hr at 37°C, following by infection of LAI virus. Blue cells were counted 2 days postinfection after staining with X-Gal. All experiments were performed in triplicate. The data are expressed as mean ± standard deviation. The number of infected cells in non-treat MAGI/CCR5 cells were 89±18 per well.

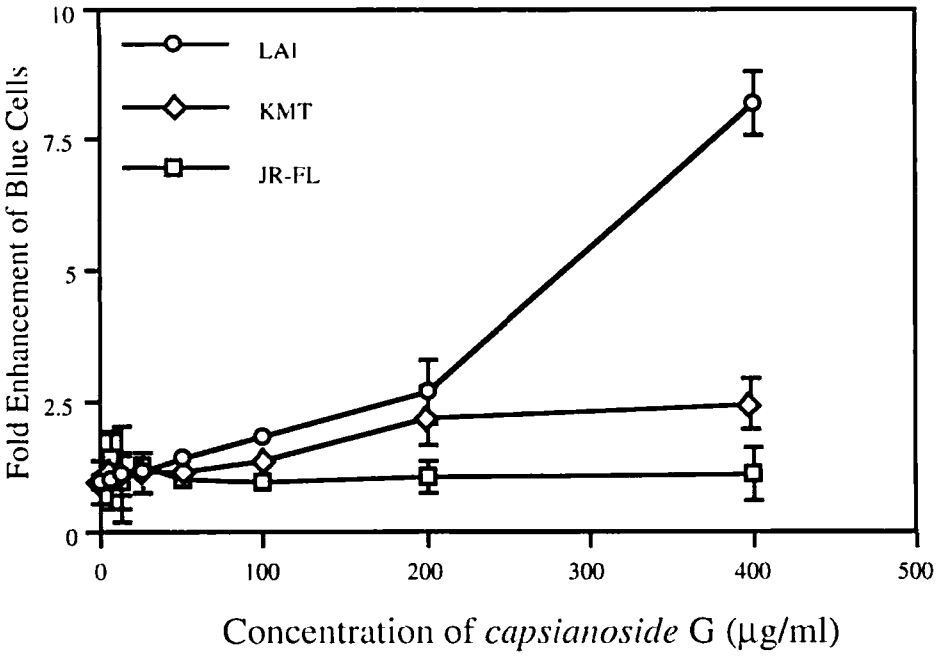

FIG. 7 A. Effect of *capsianoside* G on HIV-1 infection. MAGI/CCR5 cells were treated with various concentrations of *capsianoside* G at 37°C for 1 hr followed by an inoculation of LAI (X4), KMT (R5X4) and JR-FL (R5) viruses. Blue cells were counted 2 days postinfection. All experiments were performed in triplicate. The data are expressed as mean ± standard deviation. The number of positive cells infected with LAI, KMT and JR-FL viruses without treatment of *capsianoside* G were 90±16, 110±13 and 107±17 per well, respectively.

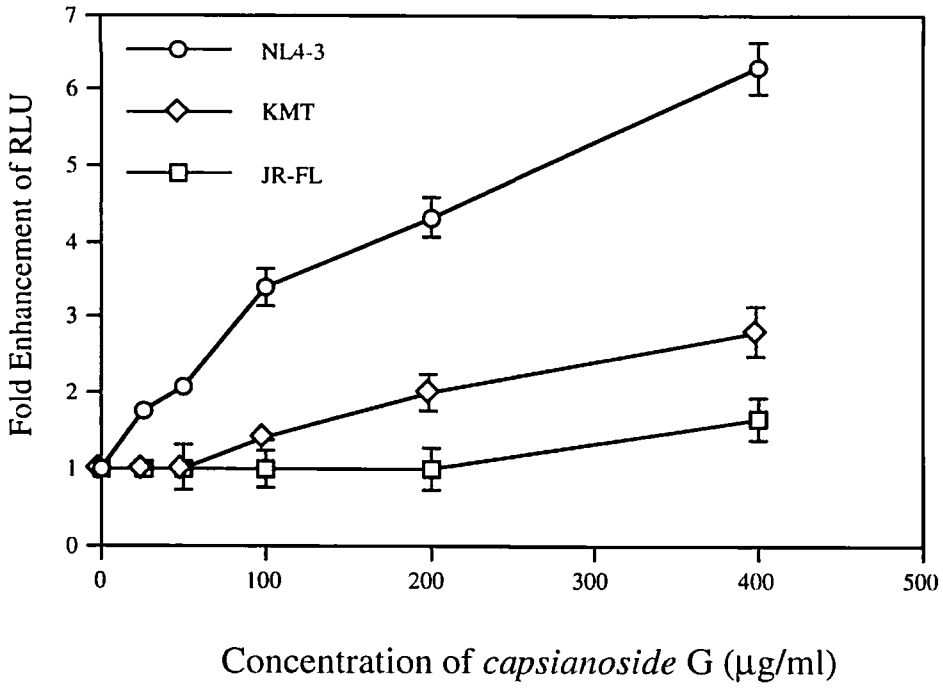

Fig. 7 B. Env-pseudotyped luciferase reporter viruses with Env from NL4-3 (X4), KMT (R5X4) and JR-FL (R5) were inoculated to various concentrations of *capsianoside* G treated MAGI/CCR5 cells. Luciferase activity were measured 2 days after infection. All experiments were performed in duplicate. The data are expressed as mean ± standard deviation. Relative light units from infected cells with NL4-3, KMT and JR-FL env-carrying pseudotyped viruses without treatment with *capsianoside* G were 982±7.4, 994±9.5 and 941±7.8, respectively.

3.3. Enhancement of R5X4 HIV-1 infection by *capsianoside* G in GHOST/CXCR4 cells

Next, to investigate whether the *capsianoside* G affected HIV-1 infection at the coreceptor level, we infected GHOST cell lines, expressing CD4 with CXCR4 (GHOST/CXCR4), or CD4 with high CCR5 expression (GHOST/Hi5), with KMT virus after cells were treated with 200 or 400 μg/ml of *capsianoside* G for 1 hr at 37°C. KMT virus infection was enhanced in GHOST/CXCR4 cells, but not in GHOST/Hi5 cells (Fig. 8 A) two days postinfection. These findings were also confirmed by using the env-pseudotyped luciferase reporter viruses in the same cell lines (Fig. 8 B).

3.4. Enhancement of X4 HIV-1 infection by *capsianoside* G in MOLT4 cells and peripheral blood mononuclear cells (PBMCs)

To determine whether *capsianoside* G also affected HIV-1 infection in T lymphocytes, we treated MOLT4 cells with 200, 300 or 400 μg/ml of *capsianoside* G for 1 hr at 37°C. Cells were washed once and infected with LAI virus for 2 hr at 37°C. After washing twice with RPMI-1640 medium, the infected cells were cultured for 10 days, while changing the

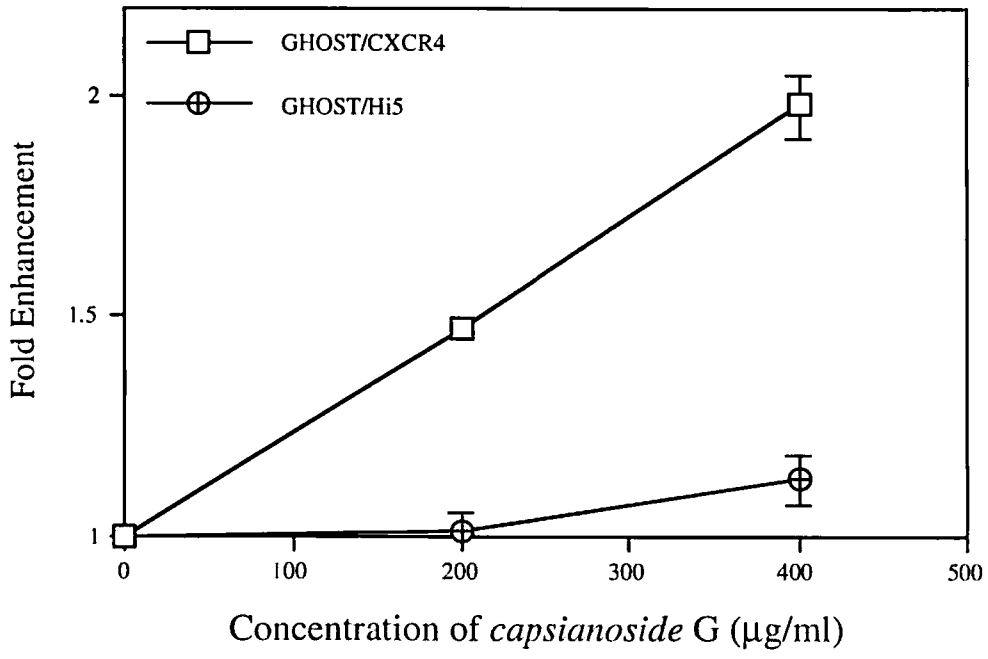

Fig. 8 A. Effect of *capsianoside* G on KMT (R5X4) infection of GHOST/CXCR4 and GHOST/Hi5 cells. Induction of GFP were detected using a FACScan flow cytometer 2 days after infection. All experiments were performed in triplicate. Without *capsianoside* G treatment, the percentage of GFP-positive cells in GHOST/CXCR4 and GHOST/Hi5 were 6.66% and 9.72%, respectively. The data are expressed as mean standard ± deviation.

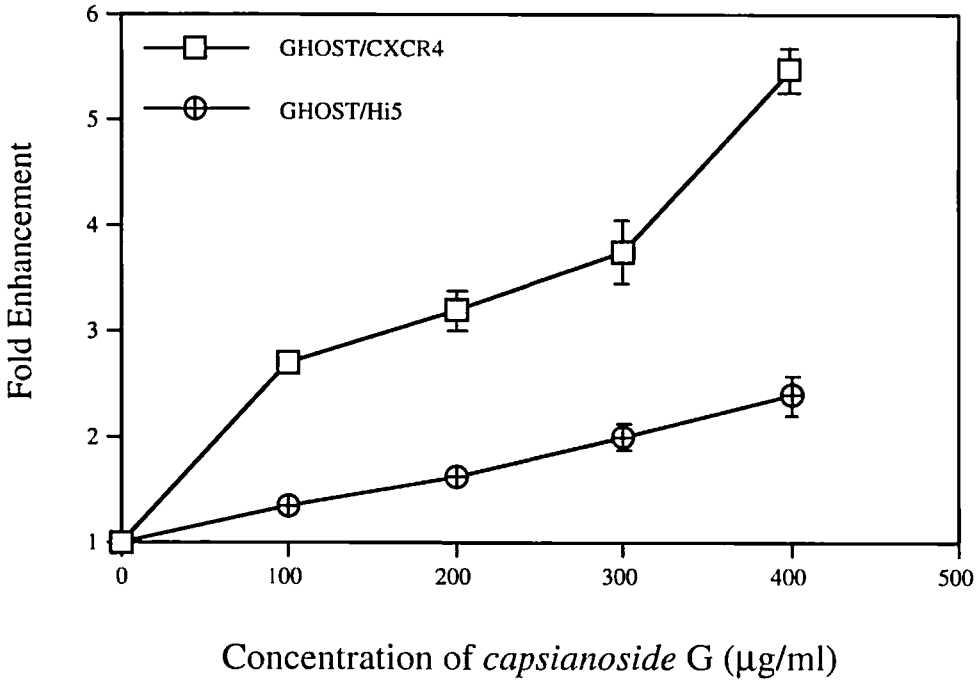

Fig. 8 B. Effect of *capsianoside* G on Env-pseudotyped luciferase reporter KMT (R5X4) infection of GHOST/CXCR4 and GHOST/Hi5 cells. Env-pseudotyped luciferase reporter viruses with Env from KMT were inoculated to *capsianoside* G pre-treated GHOST/CXCR4 and GHOST/Hi5 cells. Luciferase activity were measured 2 days after infection. All experiments were performed in duplicate. The data are expressed as mean standard ± deviation.

medium and periodically sampling the supernatants. The amount of HIV-1 produced from the infected cells was determined by measuring the amount of p24 antigen in the supernatants. The virus was released 6.3-fold more in MOLT4 cells treated with 400 μg/ml of *capsianoside* G than in controls and in cells treated with 200 or 300 μg/ml of *capsianoside* G (Fig. 9 A). Similar experiments were done using PHA-stimulated PBMC and purified macrophages. We found 20-fold enhancement by 400 μg/ml of *capsianoside* G at 7 days postinfection (Fig. 9 B), although toxicity of the treated cells was slightly discerned at this high dose. However, no effect was observed in the case of macrophages (data not shown).

3.5. Effect of *capsianoside* G on receptor expression of target cells, viral adsorption and transcription

To examine whether enhancement of the HIV-1 infection was due to the increased expression of receptors, the expression level of CD4, CXCR4 or CCR5 on *capsianoside* G-treated MAGI/CCR5 cells (200 and 400 μg/ml) was measured by FACScan flow cytometry. No significant change of CD4, CXCR4 and CCR5 was observed in terms of the expression level in MAGI/CCR5 cells (Fig. 10) as well as GHOST and MOLT4 cells (data not shown).

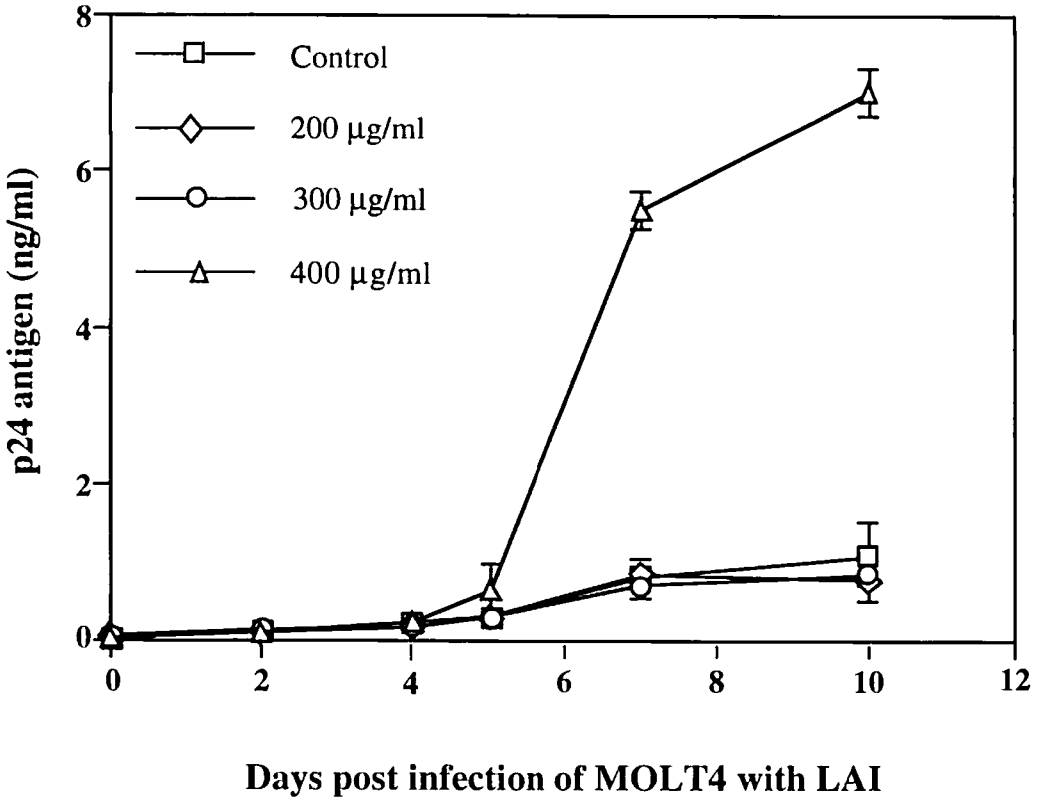

FIG. 9 A. Effect of *capsianoside* G on HIV-1 infection in MOLT4 cells. MOLT4 cells were treated with *capsianoside* G for 1h before LAI virus infection. The viruses produced from the culture supernatant at indicated time were measured by the p24 antigen ELISA. All experiments were performed in duplicate. The data are expressed as mean ± standard deviation.

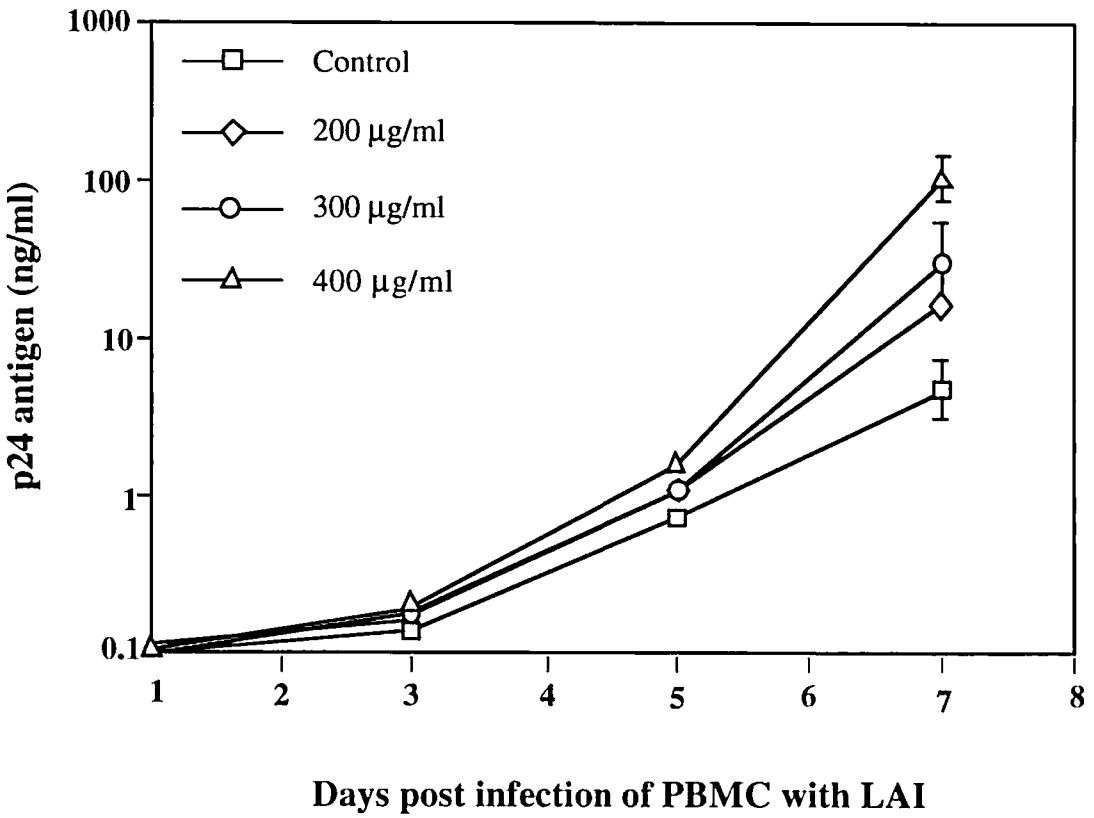

FIG. 9 B. Effect of *capsianoside* G on HIV-1 infection in PBMC. PBMC were treated with *capsianoside* G for 1hr before LAI virus infection. The viruses produced from the culture supernatant at indicated time were measured by the p24 antigen ELISA. All experiments were performed in duplicate. The data are expressed as mean ± standard deviation.

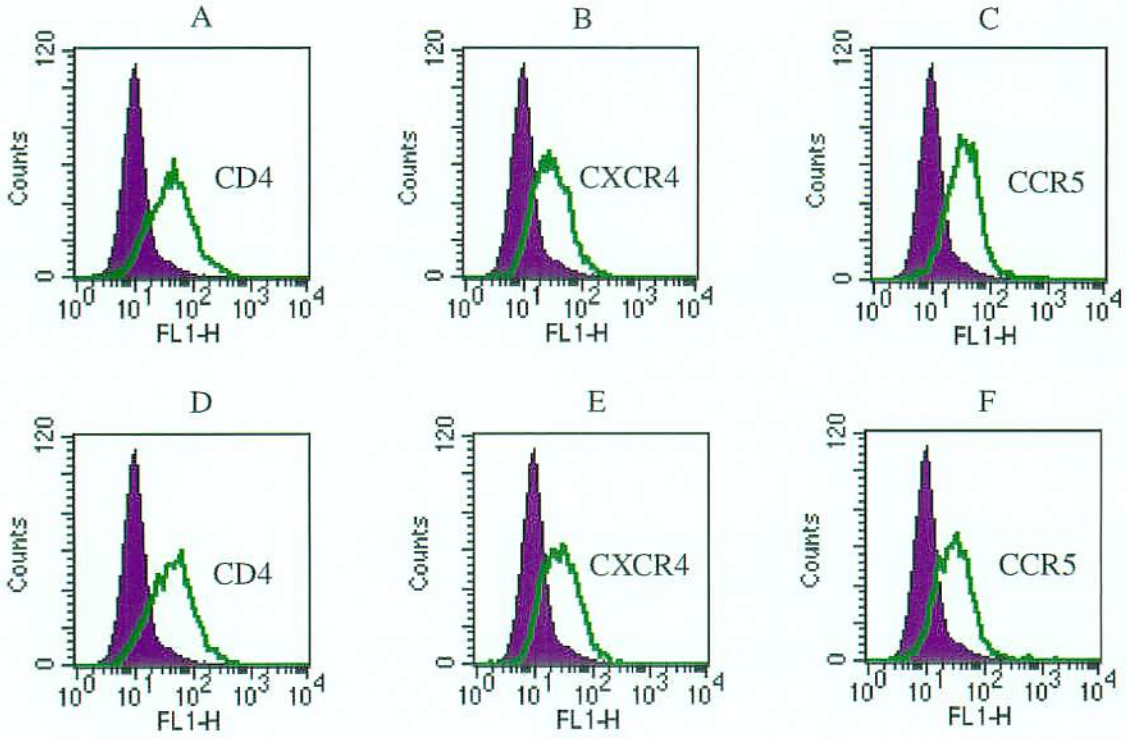

Fig. 10. Effect of *capsianoside* G on expression of CD4, CXCR4, and CCR5 on MAGI/CCR5 cells. MAGI/CCR5 cells were treated with (D, E, and F) or without (A, B, and C) *capsianoside* G (400 μg/ml) at 37°C for 1 hr. The Leu3a was used for detecting CD4 (A and D), 12G5 for CXCR4 (B and E) and 2D7 for CCR5 (C and F).

Next we quantified the amount of LAI virus adsorbed on pretreated MAGI/CCR5 cells (with 100, 300 or 400 μg/ml of *capsianoside* G) after incubating with LAI virus for 1hr at 37°C. Cells were then washed and lysed to measure the amount of p24 antigen level. *Capsianoside* G did not increase the binding of HIV-1 viruses to the cells, but rather decreased lightly the level in a dose-dependent manner (Fig. 11).

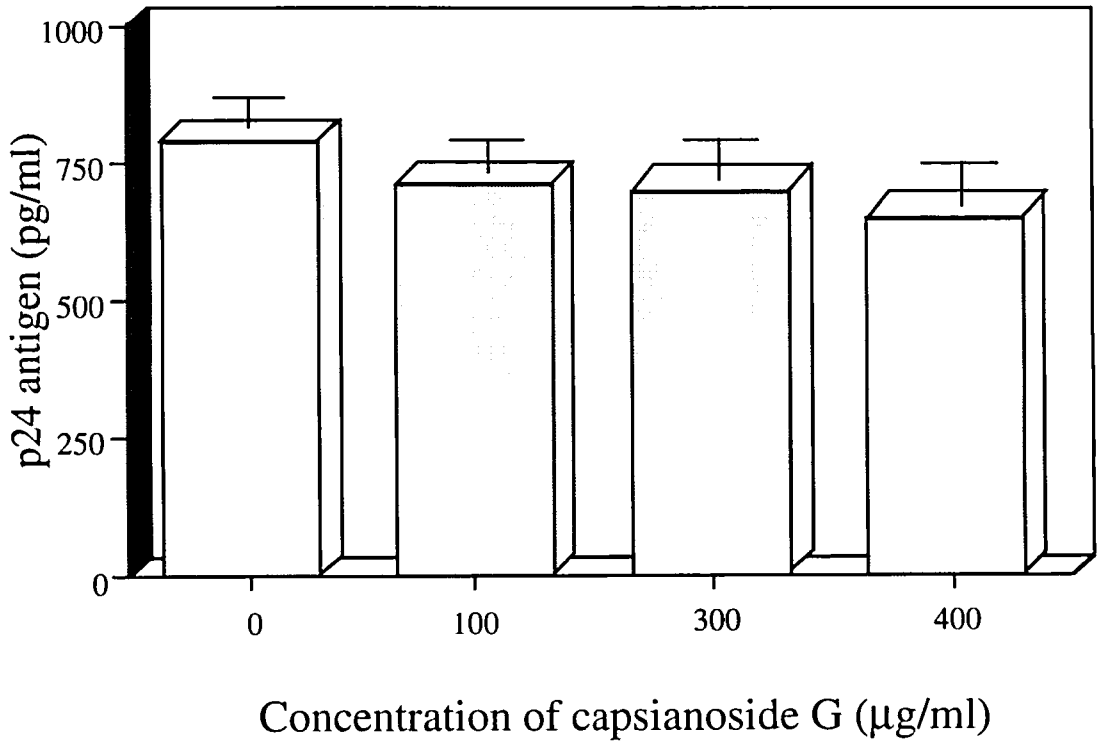

Fig. 11. Effect of *capsianoside* G on LAI virus adsorption. Bar shows standard deviation of triplicate experiments.

To clarify whether *capsianoside* G affected the transcription of proviral HIV-1 DNA, we treated chronically infected MOLT4LAI cells (100% HIV-1 antigen-positive) with 400 μg/ml of *capsianoside* G or 1 ng/ml of 12-O-tetradecanoylphorbol-13-acetate (TPA) (Sigma) as a positive control for 1 hr at 37°C. Cells were washed and cultured for 2 days followed by measurement of p24 antigen in the culture supernatants. The level of p24 antigen was increased by TPA and not by *capsianoside* G (Fig. 12). This suggested that the enhanced HIV-1 infectivity by *capsianoside* G was not responsible for the increased level of transcription of proviral HIV-1 DNA.

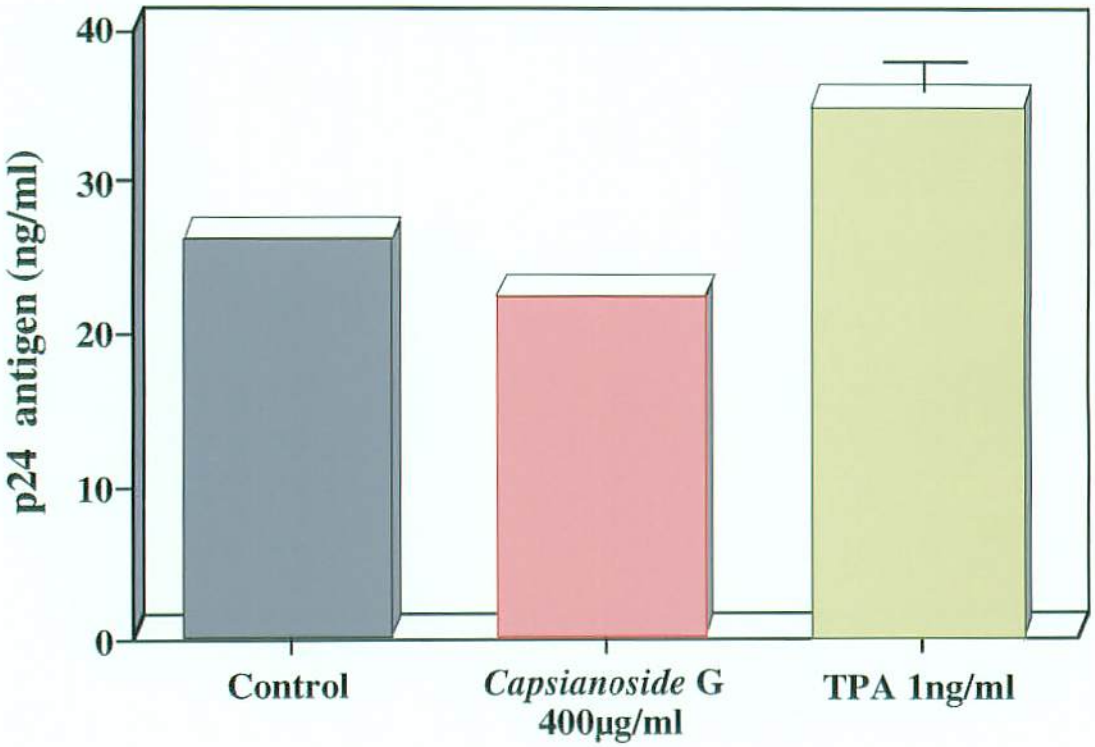

Fig. 12. Effect of *Capsianoside* G and TPA on reactivation of proviral HIV-1 DNA of MOLT-4LAI chronically infected cells

3.6. Effect of *capsianoside* G on capping and colocalization of CD4 and CXCR4

Laser scanning confocal microscopic analyses was performed to observe the localization of CD4, CXCR4 and CCR5 on MAGI/CCR5 cells. In *capsianoside* G non-treated cells, we observed that CD4, CXCR4 and CD4/CXCR4 were homogeneously distributed on the cell surface (A, B and C, respectively, in Fig. 13). When cells were treated with 400 μg/ml of *capsianoside* G at 37°C for 1 hr, the compound induced capping and colocalization of CD4 and CXCR4 receptors in a polarized pattern (Fig. 13 F). The CD4/CXCR4-capped and colocalized cells were 16.0% out of total *capsianoside* G-treated MAGI/CCR5 cells, however, they were 1.3% in non-treated cells. The CD4/CCR5-capped and colocalized cells were less than 1% in both treated and non-treated MAGI/CCR5 cells (data not shown). Treatment of the cells with *capsianoside* G at 4°C for 1 hr showed no colocalization or capping of CD4 and CXCR4 (data not shown).

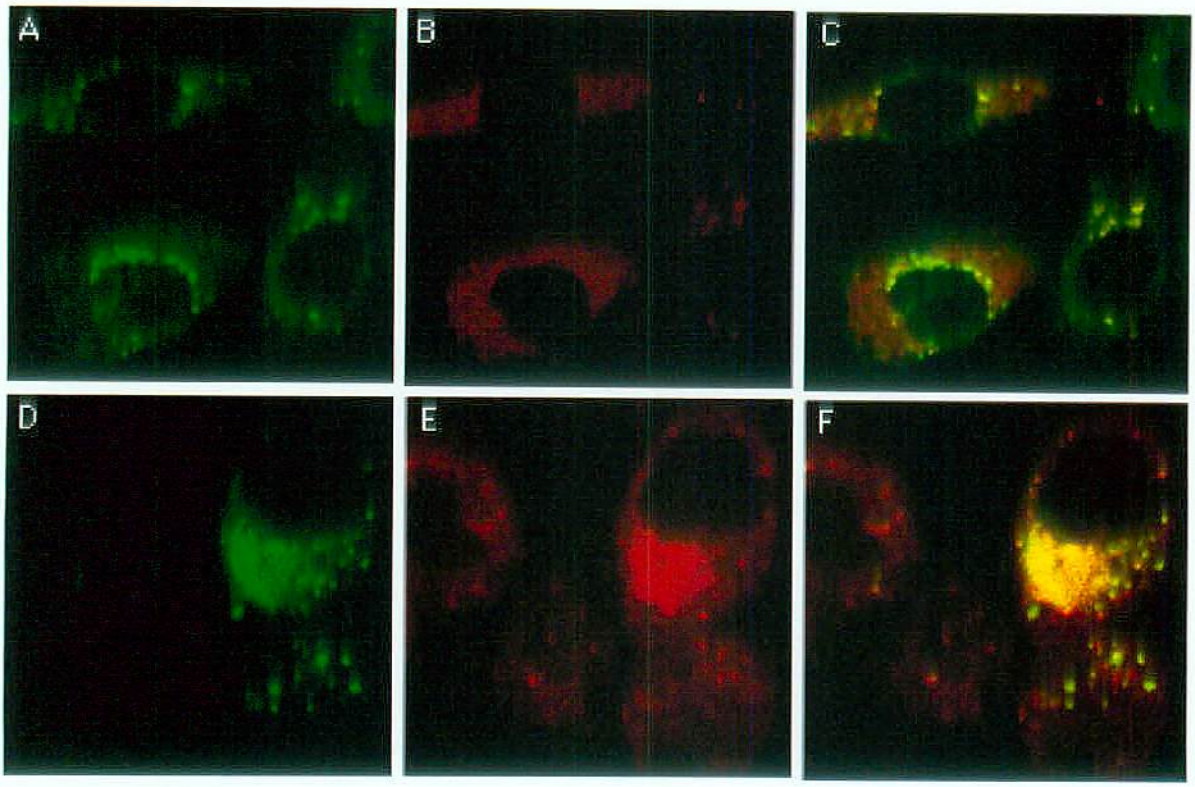

FIG. 13. Effect of *capsianoside* G on localization of CD4 and CXCR4 on MAGI/CCR5 cells. Cells were untreated (A, B and C) or treated with *capsianoside* G (400 µg/ml) (D, E and F) at 37°C for 1 hr. The cells were stained with anti-CD4 mAb conjugated with FITC (A and D, visualized as green) and anti-CXCR4 mAb plus secondary antibody conjugated with rhodamine (B and E, visualized as red). Colocalization of CD4 and CXCR4 is illustrated as yellow (C and F).

DISCUSSION

4. DISCUSSION

Recognizing the causative agent of AIDS as a retrovirus led to an immediate emphasis on arresting the replicative cycle of the HIV-1 (65). Many researchers have been working on finding compounds which could suppress HIV-1 infection. In response to highly active antiretroviral therapy (HAART), plasma HIV-1 RNA falls to below detection level in most the therapy-native patients fully adherent to prescribed medication (66~68). This virological response translates into a significant delay in clinical progression and a reduction in mortality(69~70), but it does not achieve the eradication of HIV (71). Therefore, searching new compound that could affect HIV-1 infection will be a long term task.

In the present study, we detected the effect of the new coupounds, *capsianosides*, on HIV-1 infection. Although the inhibitory infectivity of these compounds was observed, the striking results was the enhancement effect of *capsianoside* G on X4 virus infection. To know enhancing mechanism of this compound could be useful for understanding the virus, target cells, and compound interaction.

Findings in the present study shed light on the mechanisms of HIV-1 infection modulated by host cell factor(s). When cells were treated with *capsianoside* G for 1 hr at 37°C before viral exposure, T-tropic (X4) HIV-1 infection was enhanced by 8-folds compared with untreated cells (Fig. 6 A). This enhancing effect was markedly less in dualtropic (R5X4) HIV-1

and almost nil in M-tropic (R5) HIV-1 (Fig. 6 A). Although *capsianoside* G had no effect on (i) the expression level of CD4, CXCR4, and CCR5, (ii) virus adsorption and (iii) proviral HIV-1 DNA transcription, yet laser scanning confocal microscopy showed capping and colocalization of CD4 and CXCR4 in *capsianoside* G-treated MAGI/CCR5 cells. However, CD4 and CCR5 were not colocalized and capped by similar treatment, which suggests why *capsianoside* G selectively enhanced X4 HIV-1 infection. In this study, we found that capping and colocalization were not induced in MAGI/CCR5 cells treated with *capsianoside* G at 4°C for 1 hr. A previous study also reported that virus entry is temperature-dependent, which is associated with both lipid bilayer mobility thermodynamics of membrane and energy-dependent cytoskeletal movement of target cells (72). *Capsianosides* have been shown to bind to the cell membrane and to stimulate actin ring reorganization at 37°C (46). Actin ring reorganization by *capsianoside* G is most probably associated with the capping and colocalization of CD4 and CXCR4, therefore enhancing the infection of X4 HIV-1 at the viral penetration step.

Four different *capsianosides* were used in the present study (Table. 2). After screening the effect of these compounds on HIV-1 infection by MAGI assay, we found that *capsianoside* G exerted the most marked effect. *Capsianoside* G is characterized by greater hydrophobicity compared with other compounds because *capsianoside* G is an ester of acyclic diterpene

glycoside with a short sugar chain. *Capsianoside* G might possess highly binding affinity to lipid-bilayered membrane of cells. The reason why c*apsianoside* G induced capping and colocalization especially of CD4 and CXCR4 is still unknown. A possible explanation is that *capsianoside* G might bind both CD4 and CXCR4 molecules and act as a crosslinker, thus stimulating the target cell membrane to induce increased membrane fluidity by which CD4 and CXCR4 are capped. The changes to the membrane might allow HIV-1 viruses to enter the cells more easily.

Heparan sulfate and poly-L-lysin have also dichotomous effects on viral infection (73, 74). Likewise, glycolipids tend to exert an opposite effect on HIV-1 infection by different methods of treatment. Although 400 μg/ml of *capsianoside* G showed an 8-fold enhancement in a dose-dependent manner, we found no obvious enhancement by *capsianosides* II, XI, and A with X4 virus (Fig. 5). The infection was inhibited by 31% when the MAGI/CCR5 cells were treated with 400 μg/ml of *capsianoside* G and X4 virus during cultivation (Table. 2). Another glycolipid, Fattiviracin A1, from *streptomyces* also inhibited the viral infection when added during virus exposure, whereas it increased the efficacy of infection when the cells were pretreated with the compound before virus exposure. A possibility is raised that the compounds interfere with virus-cells fusion by affecting the hydrophobic regions of gp41 if adding the compounds during infection.

Although CD4/CXCR4 colocalization has been demonstrated to occur when cells are treated with HIV-1 gp120 (75, 76), the association of this phenomenon with viral infection remains unclear. The idea that not only *capsianoside* G but also gp120 could induce the colocalization of CD4 and CXCR4 suggests that the colocalization of both molecules may be essential for the viral penetration and infection. Recent report (77) showed that the primary determinant of the inefficient infection of macroghages by X4 viruses was the relatively low level of CXCR4 expressed on the surface of these primary cells in culture. They proposed that the cell tropisms displayed by different strains of HIV-1 in culture can largely be explained on the basis of differential requirements for cell surface CD4 and/or coreceptor expression levels. In this regard, colocalization of CD4 and CXCR4 could be the chance of enhanced susceptibility of the X4 virus infection, if one virion required multiple bindings of gp120 with receptors for penetration into cell.

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