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Peltophorum Africanum, a Traditional South African Medicinal Plant, Contains an Anti HIV-1 Constituent, Betulinic Acid

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The biodiversity of medicinal plants in South Africa makes them rich sources of leading compounds for the development of novel drugs. *Peltophorum africanum* (Fabaceae) is a deciduous tree widespread in South Africa. The stem bark has been traditionally employed to treat diarrhoea, dysentery, sore throat, wounds, human immunodeficiency virus/ acquired immune deficiency syndrome (HIV/AIDS), venereal diseases and infertility. To evaluate these ethnobotanical clues and isolate lead compounds, butanol and ethyl acetate extracts of the stem bark were screened for their inhibitory activities against HIV-1 using MAGI CCR5+ cells, which are derived from HeLa cervical cancer cells and express HIV receptor CD4, a chemokine receptor CCR5 and HIV-LTR-β- galactosidase. Bioassay-guided fractionation using silica gel chromatography was also conducted. The ethyl acetate and butanol extracts of the stem bark of *Peltophorum africanum* showed inhibitory activity against HIV-1, CXCR4 (X4) and CCR5 (R5) tropic viruses. The ethyl acetate and butanol extracts yielded previously reported anti-HIV compounds, (+)-catechin, a flavonoid, and bergenin, a C-galloylglycoside, respectively. Furthermore, we identified betulinic acid from the ethyl acetate fraction for the first time. The fractions, which contained betulinic acid, showed the highest selective index. We therefore describe the presence of betulinic acid, a not well-known anti-HIV compound, in an African medicinal herb, which has been used for therapy, and claim that betulinic acid is the predominant anti-HIV-1 constituent of *Peltophorum africanum*. These data suggest that betulinic acid and its analogues could be used as potential therapeutics for HIV-1 infection. ——— South Africa; Medicinal plants; HIV-1; Extracts; MAGI CCR5+ cells.

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Human immunodeficiency virus (HIV) is currently one of the most serious infectious pathogens with devastating consequences. HIV is the causative agent of acquired immune deficiency syndrome (AIDS) (Barre-Sinoussi et al. 1983; Gallo et al. 1983) and is now pandemic. In 2005, UNAIDS estimated that 40 million people were infected worldwide, with 25 million in sub-Saharan Africa, while approximately 2.4 million deaths were attributed to AIDS in 2005. Southern Africa represents one of the sub regions hardest hit by HIV. Based on antenatal seroprevalence surveys, infection levels surpass 30 percent in Botswana, Lesotho, Malawi, South Africa and Swaziland, with Angola, Mozambique, Namibia, Zambia, and Zimbabwe having 5, 15, 18, 16 and 25 percent, respectively (UNAIDS. 2005; Bessong and Obi 2006).

Highly Active Antiretroviral Therapy (HAART) is an effective form of treatment against HIV but is inadequate due to drug resistance (Gulick et al. 1997; Perelson et al. 1997; Francois and Hance 2004). Screening of plant extracts for antiretroviral activity is important because plant-derived anti-HIV compounds can inhibit replication of the virus by interfering with one or more of the ten steps of the HIV replicative cycle (Vlietinck et al. 1998; De Clercq 2000; Kong et al. 2003). The anti-HIV active compounds of plant origin possess diverse chemical structures, e.g. glycyrrhizin which has been studied in AIDS patients (Ito et al. 1988; Hattori et al. 1989). It is evident therefore that plants can be useful sources or leads for the discovery of

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novel anti-HIV compounds.

The use of medicinal plants for treating HIV/AIDS and other opportunistic infections of bacterial, fungal, protozoan or viral etiology is widespread in South Africa. Patients seek medical attention from traditional healers for many reasons including financial and traditional ones (Morris 2002). In the case of HIV/AIDS, medicinal plants function by the direct inhibition of virus replication, boosting of the immune system, or having inhibitory properties against one or several opportunistic infections (Lall and Meyer 2001; Obi et al. 2002; Lall et al. 2003; Obi et al. 2003). However, information on the potential therapeutic effects of South African medicinal plants on HIV infection is still lacking. Previous studies by Bessong and Obi have shown that medicinal plants from South Africa have strong anti HIV-1 activity in vitro against reverse transcriptase and integrase (Bessong et al. 2005). In the present study, we therefore investigated the anti-HIV activity of extracts from the stem bark of *Peltophorum africanum*. We also attempted to isolate the lead compounds from these medicinal plant extracts. We demonstrated that *Peltophorum africanum* contains three lead compounds. However, the amount of the compounds may limit their clinical efficacy.

Materials and Methods

Selection of plants

The selection of plants was based on information from traditional healers.

Identification and collection of plants

The identification and preservation of plants collected at Vuwani, Vhembe district, Limpopo, South Africa were done by a botanist, Mr. P. Tshisikhawe, Department of Biological Sciences, University of Venda, South Africa.

Drying of plants

Plant materials were washed with distilled water and dried in an oven (37°C) for two weeks. They were then chopped into small pieces and ground (scientific blender) to a fine powder.

Preparation of crude extracts

Crude extracts were prepared according to Bessong et al. 2004. Briefly, 200 g of powdered material were soaked in 1 L of methanol and left overnight on a Labcon platform shaker. The soaked plant material was filtered through a cheese cloth, and then through a funnel with qualitative Whatman filter paper No. 3 (W&R, England, UK). The residue was extracted as described previously. The filtrates were then evaporated to dryness using a rotary evaporator (RE 200, Bibby Sterillin, LTD, UK) at 40°C. The crude extracts were stored in the dark until further use.

Fractionation and Isolation of Compounds

Fractionation of the extract of Peltophorum africanum

This extract was then partitioned with ethyl acetate (200 mL × 3) and water (200 mL) to yield ethyl acetate solubles (3.08 g) and a water layer, which was extracted with *n*-butanol (200 mL × 3) to afford n-butanol solubles (6.33 g). The ethyl acetate solubles were chromatographed over silica gel 60 (70-230 mesh, Merck), and the column was eluted with hexane-ethyl acetate and ethyl acetate-methanol by increasing the polarity to give five fractions, PA-e-1 (161.2 mg), PA-e-2 (140.6 mg), PA-e-3 (565.1 mg), PA-e-4 (1517.7 mg) and PA-e-5 (451.9 mg). The *n*-butanol solubles were also chromatographed over silica gel to give six fractions, PA-b-1 (16.4 mg), PA-b-2 (861.3 mg), PA-b-3 (426.6 mg), PA-b-4 (3436.2 mg), PA-b-5 (608.0 mg) and PA-b-6 (47.4 mg).

Identification of compounds

PA-e-2 was further chromatographed over silica gel twice (elutant: chloroform-methanol (49 : 1) and toluene-ethyl acetate (19 : 1), respectively) to give crude betulinic acid (49.6 mg), which was recrystallized from chloroform-methanol (1 : 1) to yield purified betulinic acid (23.5 mg) (Fig. 1). PA-e-4 was also chromatographed over silica gel three times (elutant: chloroform-methanol (4 : 1)) to give (+)-catechin 3-*O*-α-L-rhamnopyranoside (1.4 mg). PA-b-2 was chromatographed over silica gel (elutant: chloroform-methanol (37 : 3)) to yield bergenin (808.5 mg).

We also used glycyrrhizin (provided by Minophagen Company, Tokyo, Japan), which is known to inhibit HIV-1 replication in vivo and in vitro (Hattori et al. 1989; Takei et al. 2005), because both betulinic acid and glycyrrhizin belong to the triterpenoid group and have similar structures.

Cell culture, and viruses

MAGI CCR5+ cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) at 37°C and 5% CO_2 supplemented with 10% heat inactivated fetal bovine serum, 200 μg/ml G418, 100 μg/ml hygromycin B and 100 μg/ml zeocin (complete medium) (Chackerian et al. 1997). Stocks of HIV-1 strain pNL4-3 (Adachi et al. 1986) (X4) and JRCSF virus (R5) (Takeuchi et al. 2002) were obtained from Drs. A. Adachi and Y. Koyanagi, respectively.

Anti HIV-1 assay

MAGI assay

MAGI CCR5+ cells are HeLa-derived cells that express HIV receptor CD4 coreceptors, CCR5 and CXCR4. This cell line contains an integrated copy of HIV-LTR fused to β-galactosidase (gal) reporter gene, and expresses β-gal in the presence of HIV tat (Chackerian et al. 1997). To estimate the anti-HIV-1 activities of the fractions, MAGI assays were performed as previously described with some modifications (Uchida et al. 1997). Briefly, MAGI CCR5+ cells in complete medium were plated (1×10^4 cells/well) and cultured in 96-well, flat bottomed microculture plates. The culture medium was then replaced with fresh medium containing various concentrations of the fractions in duplicate. These fractions were suspended in dimethyl sulfoxide (DMSO) at a concentration of 50 mg/ml and then serially diluted with complete medium. The diluted virus was added 30 min later. The HIV-infected (10 ng of p24) or mock-infected MAGI CCR5+ cells were cultured with the extracts continuously present and no cell washing was performed throughout the culture. Cells were stained with chlorophenolred β-D-galactopyranoside (CPRG) as previously described (Felgner et al. 1993; Flosh et al. 1998). After 3 days of culture, the medium was removed and the cells were lyzed with 100 μl of phosphate buffered saline (PBS) containing 1% Triton X-100 for 30 min at room temperature. The cells were then incubated for 60 min at 37°C with 100 μl of a staining solution containing 0.01 M KH_2PO_4, 0.1 M K_2HPO_4, 2 mM $MgCl_2$ and 10 mM CPRG. In cells infected with

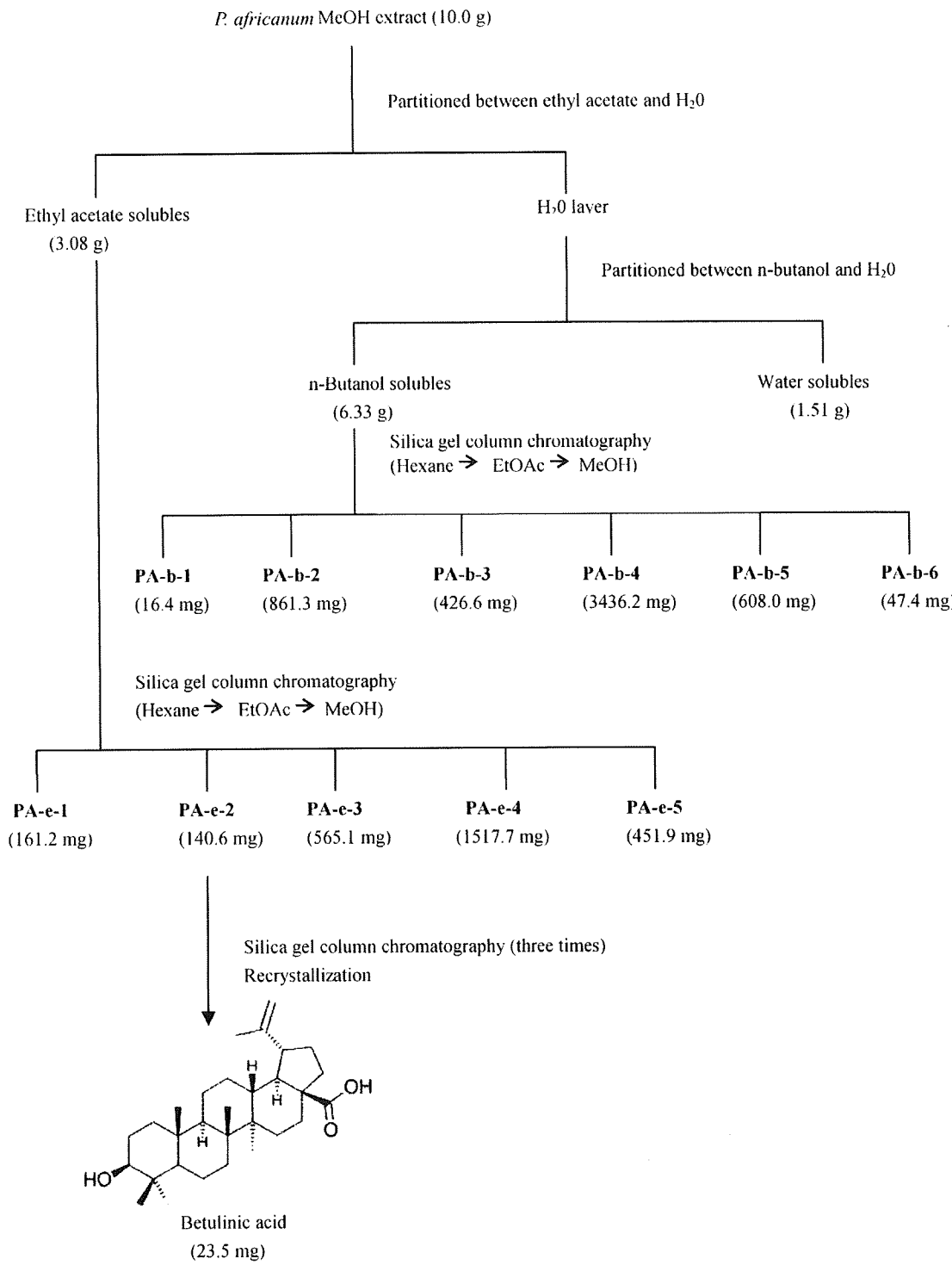

Fig.1. Fractionation procedures of *Peltophorum africanum*.
Bioassay fractionation of various fractions from the extracts (10.0 g) of *Peltophorum africanum* was performed using silica gel chromatography. n-Butanol solubles yielded fractions PA-b-1 to PA-b-6 whereas ethyl acetate solubles yielded fractions PA-e-1 to PA-e-5. Fraction PA-e-2 was chromatographed twice over silica gel to yield crude betulinic acid (49.6 mg), followed by further recrystallisation to yield purified betulinic acid (23.5 mg).

HIV, the integrated HIV-LTR-β-gal reporter gene was expressed and the cells turned from yellow to brown on staining. Absorbances from the 96 well plates were then read at 570 nm in a microplate reader (Vmax, Molecular Devices, Sunnyvale, CA).

Cytotoxicity assay

Cytotoxic assays were performed as follows using cell counting kit-8 (Dojindo Com, Kumamoto, Japan). MAGI CCR5+ cells in complete medium were cultured in 96-well microculture plates at 1×10^4 cells/well for 24h. The culture medium was then replaced with fresh medium containing fractions in duplicate. After 3 days, cell viability was quantified according to the company's instructions.

The 50% inhibition concentration (IC_{50}) of the herbs was determined from a curve relating the percentage inhibitory activities to the concentration of the herbs. The 50% cytotoxic concentration (CC_{50}) was calculated in a similar manner. The selectivity index (SI) was calculated as CC_{50}/IC_{50} (Baba et al. 1987).

Results

The *Peltophorum africanum* methanol fractions were examined. The methanol fraction showed anti HIV-1 activity with R5 virus with an SI of 3. The ethyl acetate fraction showed anti HIV-1 activity with the X4 virus with an SI of 189. The butanol fraction showed anti HIV-1 activity with the R5 virus with an SI of 37 and the X4 virus with an SI of 11 (Table 1).

The butanol fraction of *Peltophorum africanum* was then examined. The fraction PA-b-1 showed anti HIV-1 activity with the R5 virus with an SI of 13.5. The fraction PA-b-4 showed the anti HIV-1 activity with X4 virus with an SI of 16.9. The fraction PA-b-5 showed high anti HIV-1 activity with R5 virus with an SI of 135 (Table 2).

The ethyl acetate fraction was then examined. The ethyl acetate fraction of PA-e-2 inhibited virus replication in MAGI CCR5+ cells with an R5 virus IC_{50} value of 1.8 μg/ml, CC_{50} of 308 μg/ml and an SI of 171.1. For the X4 virus, the IC_{50} value was 1 μg/ml, CC_{50} was 308 μg/ml and an SI was 308. This fraction showed high anti-HIV-1 activity and it is from this fraction that betulinic acid was isolated. The SI value for fraction PA-e-5 using the X4 virus was 98 (Table 3). The fractionation of *Peltophorum africanum* is shown in Fig. 1. An ethyl acetate extract (PA-e-2) of the dry powdered stem bark of *Peltophorum africanum* on concentration yielded crude betulinic acid (49.6 mg), which was filtered out, with repeated chromatography (silica gel) of the filtrate, and recrystallization led to the isolation of purified betulinic acid (23.5 mg). Similarly, repeated chro-

Table 1. Anti-HIV-1 activity and cytotoxicity of fractions isolated from *Peltophorum africanum*.

Fraction no.	CC_{50}[a] (μg/mL)	IC_{50}[b] (μg/mL)		*SI*[c] (CC_{50}/IC_{50})	
		R5[d]	X4[e]	R5[d]	X4[e]
Methanol	255	78	ND	3	ND
Ethyl acetate	378	4	2	94.5	189
Butanol	110	3	10	37	11
Water	125	1	8	125	15.6

CC_{50}[a] = Concentration which inhibits MAGI CCR5+ cell growth by 50%. IC_{50}[b] = Concentration which inhibits virus replication by 50%. *SI*[c] = Selective index. *R5*[d] = R5 HIV-1 JRCSF. *X4*[e] = X4 HIV-1 pNL4-3. *ND* = Not determined.

Table 2. Anti-HIV-1 activity and cytotoxicity of fractions isolated from *Peltophorum africanum* butanol fraction.

Fraction no.	CC_{50}[a] (μg/mL)	IC_{50}[b] (μg/mL)		*SI*[c] (CC_{50}/IC_{50})	
		R5[d]	X4[e]	R5[d]	X4[e]
PA-b-1	100	7.4	64	13.5	1.5
PA-b-2	251	86	109	2.9	2.3
PA-b-3	489	199	41	2.45	11.9
PA-b-4	< 83	29.0	4.9	< 2.86	< 16.9
PA-b-5	756	5.6	68	135	11.1
PA-b-6	100	31	45	3	2.2

CC_{50}[a] = Concentration which inhibits MAGI CCR5+ cell growth by 50%. IC_{50}[b] = Concentration which inhibits virus replication by 50%. *SI*[c] = Selective index. *R5*[d] = R5 HIV-1 JRCSF. *X4*[e] = X4 HIV-1 pNL4-3.

Table 3. Anti-HIV-1 activity and cytotoxicity of fractions isolated from *Peltophorum africanum* ethyl acetate fraction.

Fraction no.	CC_{50}[a] (μg/mL)	IC_{50}[b] (μg/mL)		*SI*[c] (CC_{50}/IC_{50})	
		R5[d]	X4[e]	R5[d]	X4[e]
PA-e-1	498	9	8.5	55.3	58.5
PA-e-2	308	1.8	1	171.1	308
PA-e-3	50	9.94	11	5	4.5
PA-e-4	1009	9.9	98	102	10
PA-e-5	127	15.2	1.3	8	98

CC_{50}[a] = Concentration which inhibits MAGI CCR5+ cell growth by 50%. IC_{50}[b] = Concentration which inhibits virus replication by 50%. *SI*[c] = Selective index. *R5*[d] = R5 HIV-1 JRCSF. *X4*[e] = X4 HIV-1 pNL4-3.

Table 4. Potent activity of betulinic acid against X4-HIV-1 and R5-HIV-1.

Anti HIV-1 activity against HIV-1 laboratory isolates and cytotoxicity using MAGI CCR5 cells

compound	MAGI (CCR5-MAGI)			Si[b]
	IC_{50} (μg/ml)[a]		CC_{50} (μg/ml)	
	$HIV\text{-}1_{NL4\text{-}3}$	$HIV\text{-}1_{JRCSF}$		
GL	0.04		1.65	41
BA	0.04		> 0.09	> 2
GL		0.58	1.65	3
BA		0.002	> 0.09	> 45

[a] The IC_{50} values were determined with the MAGI assay. Cytotoxicity of the test compounds was also determined with the cell counting kit 8. [b] SI, selectivity index, CC_{50}/IC_{50}. BA, betulinic acid and GL, glycyrrhizin.

matography of the ethyl acetate extract of *Peltophorum africanum* PA-e-4 yielded catechin and PA-b-2 yielded bergenin.

We tested betulinic acid and glycyrrhizin against HIV-1_{NL4-3}(X4 HIV-1) and HIV-1_{JRCSF} (R5 HIV-1) using MAGI-CCR5 cells as target cells. As assessed in the MAGI assay, the IC_{50} using HIV-1_{NL4-3} was 0.04 μg/ml for betulinic acid and 0.04 μg/ml for glycyrrhizin (Table 4). In the same antiviral assay using HIV-1_{JRCSF}, the IC_{50} value was 0.002 μg/ml for betulinic acid and 0.58 μg/ml for glycyrrhizin. The CC_{50} determined using cell counting kit 8, was > 0.09 μg/ml for betulinic acid and 1.65 μg/ml for glycyrrhizin.

Discussion

There has been a trend towards using traditional medicines to treat various diseases, especially in developing countries. Medicinal plant extracts are chemically complex and diverse. They could provide a safer and more effective platform for newer drugs and could lead to better success than routine random screening. Botanical extracts provide a wide spectrum of biological and pharmacological properties, including cytoprotective, anti cancer, anti-inflammatory, immunomodulative and anti-infectious activities (Wang et al. 2006) Many compounds with anti-HIV effects have been screened out from botanicals and found to inhibit HIV at nearly all stages of the viral life cycle.

In the present study, extracts of *Peltophorum africanum* were shown to have anti-HIV activities, especially fraction PA-e-2. It is interesting to note that we could isolate the inhibitory molecule betulinic acid from the high active fraction, PA-e-2. Our study shows that betulinic acid inhibits both X4-tropic virus and R5-tropic virus, suggesting that betulinic acid is a potentially useful inhibitor of different HIV-1 strains. The betulinic acid inhibitory activity was found on HIV-1 IIIB (X4 tropic strain) infection to H9 cells; the EC_{50} value was 1.4 μM and CC_{50} was 13 μM with an SI of 9.3 (Fujioka et al. 1994).

The possible mechanism of action of betulinic acid has recently been elucidated using betulinic acid derivatives (BAD) that act as HIV-1 entry inhibitors. BAD are synthesized using betulinic acid as a scaffold and their viral targets depend on where their side chains reside. One such BAD, IC9564, inhibits HIV-1 by targeting the V3 loop of gp120, and it competed with the binding of V3-specific monoclonal antibodies 447-52D and 39F to HIV-1 IIIB (X4) gp120. IC9564 inhibited the binding of these monoclonal antibodies to gp120 in a dose-dependent manner. Since IC9564 can inhibit both R5 and X4 viruses, it also competed effectively with the binding of 447-52-D to HIV-1 Ba-L gp120 (R5). The binding of BAD likely interferes with the interactions of gp120 and the chemokine receptors. IC9564 at 5 μg/ml strongly inhibited the binding of HIV-1 Ba-L gp120/CD4 (R5) or HIV-1 IIIB gp120/CD4 (X4) complexes to HOS.CCR5 or HOS.CXCR4 cells. Additionally, Clade C viruses, which account for approximately 50% of all HIV-1 isolates around the world and are predominant in sub-Saharan Africa, are sensitive to the BAD at submicromolar concentrations. Clade C viruses were more sensitive to the inhibition of A43-D, which reduced clade C virus infection by 50% (IC_{50}) at an average concentration of 0.206 μM (Lai et al. 2008).

Several triterpene compounds have been described to have HIV-1 antiviral activity. In a previous report, glycyrrhizin inhibited the R5 HIV replication in cultures of fresh peripheral blood monocytes treated with 1-methyladenosine (PBM/MA) in a dose dependent manner. In GL treated fresh PBM/MA (100 μg/ml), 85% of HIV replication was inhibited. (Takei et al. 2005). Previous studies done in our lab have also shown that glycyrrhizin inhibits the binding of anti-CCR5 monoclonal antibody 2D7 to target cells (unpublished data).

It should also be noted that betulinic acid probably acts as a maturation inhibitor by inhibiting viral assembly/viral budding. In a study by Zhou et al. (2004), dimethylsuccinyl-betulinic acid (DSB), a derivative of betulinic acid, inhibited HIV-1 replication and functioned at a late stage of the virus life cycle but did not inhibit the HIV-1 protease in vitro or interfere with virus particle assembly or release. DSB specifically delayed the cleavage of Gag between the capsid and p2, resulting in delayed formation of the mature viral core and reduced HIV-1 infectivity (Zhou et al. 2004; Aiken and Chen 2005).

Bessong et al. (2005) reported that several active compounds could be isolated from the extracts of *Peltophorum africanum*. Using a radioactive cell-free assay, (+)-catechin, a flavonoid, was isolated from the stem bark of *Peltophorum africanum*, and shown to inhibit the 3'-end processing activity of HIV-1 IN by as much as 65% at 100 μM, but had no activity on HIV-1 RT (Bessong et al. 2005). Derivatives of catechin such as epicatechin and epigallocatechin gallate have been shown to inhibit HIV-1 RT in the micromolar range (Tillekeratne et al. 2002).

Bergenin, a C-galloylglycoside was also isolated from *Peltophorum africanum* in the present study. Bergenin has been previously isolated from *Peltophorum africanum* (Mebe and Makuhunga 1992) and several other plants, namely the roots of *Astilbe thumbergii* (Han et al. 1998) and the aerial parts of *Fluggea virosa* (Pu et al. 2002). In another study, bergenin had no activity against the RDDP and RNase H of HIV-1 RT and the 3'-end processing activity of HIV-1 IN, even at a concentration of 500 μM (Bessong et al. 2005). Elsewhere, bergenin showed weak anti-HIV-1 activity in C8166 cells infected with HIV-1_{MN} (X4 virus) with an EC_{50} value of 40 μg/ml. Further experiments showed that bergenin inhibited the binding of GP120 to sCD4 in a dose-dependent manner (Piacente et al. 1996).

It is worth noting that betulinic acid and its analogues have been used for the treatment of various infections for decades and have an acceptable oral bioavailability (Bessong et al. 2005). However, incidents of poisoning have been reported (Hamouda et al. 2000). Because of the low amount of betulinic acid in *Peltophorum africanum*, large doses of

crude extracts of the stem bark need to be consumed. The clinical efficacy of betulinic acid in *Peltophorum africanum* should therefore be carefully evaluated, because our knowledge of the toxicity profiles in humans upon administration of this medicinal plant is limited. These data suggest that betulinic acid and its analogues could be used as potential therapeutics for HIV-1.

Conclusion

A systematic evaluation against HIV-1 of one selected traditionally used South African medicinal plant was done. *Peltophorum africanum* contains an anti-HIV-1 constituent, betulinic acid, which inhibits HIV-1. There is therefore a rationale for the traditional use of the stem bark of *Peltophorum africanum* in treating HIV/AIDS and related opportunistic infections.

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References

Adachi, A., Gendelman, H.E., Koenig, S., Folks, T., Willey, R., Rabson, A. & Martin, M.A. (1986) Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J. Virol.*, **59**, 284-291.

Aiken, C. & Chen, C.H. (2005) Betulinic acid derivatives as HIV-1 antivirals. *Trends Mol. Med.*, **11**, 31-36.

Baba, M., Konno, K., Shigeta, S. & De Clercq, E. (1987) In vitro activity of (S)-9-(3-hydroxy-2-phosphonylmethoxypropyl) adenine against newly isolated clinical varicella-zoster virus strains. *Eur. J. Clin. Microbiol.*, **6**, 158-160.

Barre-Sinoussi, F., Chermann, J.C., Rey, F., Nugeyre, M.T., Chamaret, S., Gruest, C., Dauguest, C., Axler-blin, C., Vézinet-Brun, F., Rouzioux, C., Rozenbaum, W. & Montagnier, L. (1983) Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science*, **220**, 868-871.

Bessong, P.O. & Obi, C.L. (2006) Ethnopharmacology of human immunodeficiency virus in South Africa- a minireview. *Afr. J. Biotech.*, **5**, 1693-1699.

Bessong, P.O., Obi, C.L., Andreola, M., Rojas L.B., Pouysegu, L., Igumbor, E., Meyer, J.J.M., Quideau, S. & Litvak, S. (2005) Evaluation of selected South African medicinal plants for inhibitory properties against human immunodeficiency virus type 1 reverse transcriptase and integrase. *J. Ethnopharmacol.*, **99**, 83-91.

Bessong, P.O., Obi, C.L., Igumbor, E., Andreola, M. & Litvak, S. (2004) In vitro activity of three South African medicinal plants against human immunodeficiency virus type 1 reverse transcriptase. *Afr. J. Biotechnol.*, **3**, 555-559.

Chackerian, B., Long, E.M., Luciw, P.A. & Overbaugh, J. (1997) Human immunodeficiency virus type 1 co-receptors participates in post entry stages of the virus replication cycle and function in SIV infection. *J. Virol.*, **71**, 3932-3939.

De Clercq, E. (2000) Current lead natural products for the chemotherapy of human immuno deficiency virus infection. *Med. Res. Rev.*, **20**, 323-349.

Felgner, J.H., Kumar, R., Sridhar, C.N., Wheeler, C.J., Tsai, J.Y., Border, R., Ramsey, P., Martin, M. & Feigner, L.P. (1993) Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. *J. Biol. Chem.*, **269**, 2550-2561.

Flosh, V., Audrezet, M.P., Guillaume, C., Gobin, E., Bolch, Le.G., Clement, J.C., Yaouanc, J.J., Abbayes, Des.H., Mercier, B., Leroy, J.P., Abgrall, J.F. & Ferec, C. (1998) Transgene expression kinetics after transfection with cationic phosphonolipids in hematopoietic non adherent cells. *Biochim. Biophys. Acta.*, **1371**, 53-70.

Francois, C. & Hance, A.J. (2004) HIV drug resistance. *N. Engl. J. Med.*, **350**, 1023-1035.

Fujioka, T. & Kashiwada, Y. (1994) Anti-AIDS agents, 11. Betulinic acid and platanic acid as anti-HIV principles from Syzigium claviflorum, and the anti-HIV activity of structurally related triterpenoids. *J. Nat. Prod.*, **57**, 243-247.

Gallo, R.C., Sarin, P.S., Gelmann, E.P., Robert-Guroff, M., Richardson, E., Kalyanaraman, V.S., Mann, D., Sidhu, G.D., Stahl, R.E., Zolla-Pazner, S., Leibowitch, J. & Popovic, M. (1983) Isolation of human T-cell leukemia virus in acquired immune deficiency syndrome (AIDS). *Science*, **220**, 865-867.

Gulick, R.M., Mellors, J.W., Havlir, D., Eron, J.J., Gonzalez, C., Mcmahon, D., Richman, D.D., Valentine, F.T., Jonas, L., Meibohm, A., Emini, E.A. & Chodakewitz, J.A. (1997) Treatment with indinavir, zidovudine, and lamivudine in adults with human immunodeficiency virus infection and prior antiretroviral therapy. *N. Engl. J. Med.*, **337**, 734-739.

Hamouda, C., Amamou, M., Thabet, H., Yacoub, M., Hedhili, A. & Bescharnia, F. (2000) Plant poisonings from herbal medication admitted to a Tunisian toxicology intensive care unit 1983-1998. *Vet. Hum. Tox.*, **42**, 137-141.

Han, L.K., Ninomiya, H., Taniguchi, M., Kimura, Y. & Okuda, H. (1998) Norepinephrine-augmenting lipolytic effectors from Astilbe thunbergii rhizomes. *J. Nat. Prod.*, **61**, 1006-1011.

Hattori, T., Ikematsu, S., Koito, A., Matsushita, S., Maeda, Y., Hada, M., Fujimaki, M. & Takatsuki, K. (1989) Preliminary evidence for inhibitory effect of glycyrrhizin on HIV replication in patients with AIDS. *Antiviral. Res.*, **11**, 255-261 13.

Ito, M., Sato, A., Hirabayashi, K., Tanabe, F., Shigeta, S., Baba, M., De Clercq, E., Nakashima, H. & Yamamoto, N. (1988) Mechanism of inhibitory effect of glycyrrhizin on replication human immunodeficiency virus (HIV). *Antiviral Res.*, **10**, 289-298.

Kong, J.M., Goh, N.K., Chia, L.S. & Chia, T.F. (2003) Recent advances in traditional plant drugs and orchids. *Acta Pharmacol. Sin.*, **24**, 7-21.

Lall, N., Das, S.M., Hazra, B. & Meyer, J.J. (2003) Antimycobacterial activity of diospyrin derivatives and a structural analogue of diospyrin against Mycobacterium tuberculosis in vitro. *J. Antimicrob. Chemother.*, **51**, 435-438.

Lall, N. & Meyer, J.J. (2001) Inhibition of drug sensitive and drug resistant strains of Mycobacterium tuberculosis by diospyrin, isolated from Euclea natalensis. *J. Ethnopharamacol.*, **78**, 213-216.

Lai, W., Ho, P., Huang, L., Li, Z., Montefiori, D. & Chen, C. (2008) Betulinic acid derivatives that target gp120 and inhibit multiple genetic subtypes of HIV-1. *J. Antimicrob. Chemother.*, **52**, 128-136.

Mebe, P.P. & Makuhunga, P. (1992) 11-p-Coumaric acid ester of bergenin from Peltophorum africanum. *Phytochemistry*, **31**, 3286-3287.

Morris, K. (2002) South Africa tests traditional medicines. *Lancet Infect. Dis.*, **2**, 319.

Obi, C.L., Potgieter, N., Masebe, T., Mathebula, H. & Molobela, P. (2003) In vitro antibacterial activity of Venda medicinal plants. *S. Afri. J. Botany.*, **69**, 199-203.

Obi, C.L., Potgieter, N., Randima, L.P., Mavhungu, N.J., Musie, E., Bessong, P.O., Mabogo, D.E.N. & Mashimbye, J. (2002) Antibacterial activity of five medicinal plants against some medically significant human bacteria. *S. Afr. J. Sci.*, **98**, 25-28.

Piacente, S., Pizza, C., De Tommasi, N. & Mahmood, N. (1996) Constituents of Ardista japonica and their in vitro anti HIV activity. *J. Nat. Prod.*, **59**, 565-569.

Perelson, A.S., Essunger, P., Cao, Y., Vesanen, M., Hurley, A., Saksela, K., Markowitz, M. & Ho, D.D. (1997) Decay characteristics of HIV-1-infected compartments during combination therapy. *Natur.*, **387**, 88-191.

Pu, H.L., Huang, X., Zhao, J.H. & Hong, A. (2002) Bergenin is the antiarrhymic principle of Fluggea virosa. *Planta Med.*, **68**, 372-374.

Takei, M., Kobayashi, M., Li, X.D., Pollard, R.B. & Suzuki, F. (2005) Glycyrrhizin inhibits R5 HIV replication in peripheral blood monocytes treated with 1-methyladenosine. *Pathobiology*, **72**, 117-123.

Takeuchi, H., Suzuki, Y., Tatsumi, M., Hoshino, H., Daar, E.S. & Koyanagi Y. (2002) Isolation and characterization of an infectious HIV type 1 molecular clone from a patient with primary infection. *AIDS Rev. Hum. Retroviruses*, **18**, 1127-1133.

Tillekeratne, L.M.V., Sherette, A., Fulmer, J.A., Hupe, D., Gabbara, S., Peliska, J.A. & Hudson, R.A. (2002) Differential inhibition of polymerase and strand-transfer activities of HIV-1 reverse transcriptase. *Bio. Med. Che. Let.*, **12**, 525-528.

UNAIDS/WHO (2005) *AIDS Epidemic Update*, UNAIDS, Geneva, Switzerland, p. 17.

Uchida, B., Maeda, Y. & Mitsuya, H. (1997) HIV-1 protease does not play a role in the early stages of HIV-1 infection. *Antiviral Res.*, **36**, 107-113.

Vlietinck, A.J., De Bruyne, T., Apers, S. & Pieters, L.A. (1998) Plant-derived leading compounds for chemotherapy of human immunodeficiency virus (HIV) infection. *Planta Med.*, **64**, 97-109.

Wang, R., Gu, Q.,Yang, L., Chen, J., Li, S. & Zheng, Y. (2006) Anti-HIV-1 activities of extracts from the medicinal plant Rhus chinensis. *J. Ethnopharmacol.*, **105**, 269-273.

Zhou, J., Yuan, X., Dismuke, D., Forshey, B.M., Lundquist, C., Lee, K.H., Aiken, C. & Chen, C.H. (2004) Small-molecule inhibition of human immunodeficiency virus type 1 replication by specific targeting of the final step of virion maturation. *J. Virol.*, **78**, 922-929.

Characterization of a CD4-independent clinical HIV-1 that can efficiently infect human hepatocytes through chemokine (C-X-C motif) receptor 4

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Objective: HIV-1 isolates are prominently CD4-dependent and, to date, only a few laboratory-adapted CD4-independent strains have been reported. Therefore, whether CD4-independent viruses may exist in HIV-1-infected patients has remained unclear. Here, we report the successful isolation of a CD4-independent clinical HIV-1 strain, designated SDA-1, from the viral quasispecies of a therapy-naive HIV-1 and *Pneumocystis jirovecii* pneumonia patient in the late-stage of AIDS with extremely low CD4 cell count (CD4 = 1/μl). We characterized this virus and further explored whether it could infect or induce pathological effects in human hepatocytes.

Design and methods: To determine coreceptor usage and CD4-independent infection, the HIV-1 envelope (Env)-pseudotypes and Env-chimeric viruses were used.

Results: SDA-1 was able to infect $CD4^-$ cell lines through either chemokine (C-X-C motif) receptor 4 or CCR5. It still maintained the ability to infect $CD4^+$ cells through multiple coreceptors of chemokine (C-X-C motif) receptor 4, chemokine (C-C motif) receptor 5, chemokine (C-C motif) receptor 3 and chemokine (C-C motif) receptor 8. Productive infection by SDA-1 was noted in both CD4-negative hepatoma cells and primary cultured human hepatocytes. Moreover, we demonstrated that SDA-1 could efficiently infect human hepatocytes on both static and mitotic phases through chemokine (C-X-C motif) receptor 4, without inducing apoptotic cell death.

Conclusion: The present study provides evidence that emergence of CD4-independent HIV-1 virus *in vivo* may occur in HIV-1-infected patients. In addition, these results shed light on the mechanisms involved in liver damage in HIV-1-infected individuals, which could have important implications concerning the range of mutability and the pathogenesis of AIDS.

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Keywords: CD4-independence, HIV-1, human hepatocytes, human hepatoma cells

Introduction

The entry of HIV-1 into target cells requires interaction of the viral envelope (Env) with CD4 and a chemokine coreceptor [1,2]. Macrophage-tropic HIV-1 viruses primarily use chemokine (C-C motif) receptor 5 (CCR5) (R5) as a coreceptor, whereas T-cell-tropic viruses use chemokine (C-X-C motif) receptor 4

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(CXCR4) (X4). Dual-tropic viruses (R5X4) use both coreceptors [3]. A few rare viruses can also use alternative coreceptors such as chemokine (C-C motif) receptor 1 (CCR1), chemokine (C-C motif) receptor 2b (CCR2b), chemokine (C-C motif) receptor 8 (CCR8), chemokine (C-X-C motif) receptor 6 (CXCR6), G protein-coupled receptor 1 (GPR1) or GPR15/Bob for entry into coreceptor-transfected $CD4^+$ cell lines [4]. Whatever the coreceptor specificity of an HIV-1 isolate, an interaction with CD4 is always the first step in a chain of events leading to fusion of the viral envelope with the cellular membrane. However, previous studies have shown that SIV [5] and HIV-2 [6] can also infect cells independently of CD4.

In contrast to SIV and HIV-2, HIV-1 CD4-independent viruses are rarely isolated. To date, only a few laboratory CD4-independent HIV-1 variants [7–10] have been reported. Therefore, whether such viruses may exist in HIV-1-infected patients has remained unclear. However, several studies [11–14] have shown that HIV-1-DNA and p24, a core HIV-1 antigen, were detected in CD4-negative cells or tissues such as brain, kidney and liver in HIV-infected individuals, suggesting the possibility that low levels of CD4-independent variants exist *in vivo*. Among such $CD4^-$ cells or tissues, liver is an important organ in determining the prognosis of HIV-1-infected patients. End-stage liver disease is becoming a frequent cause of death in HIV-1-infected hospitalized patients [15–17]. Although the cause of liver injury in HIV-1 patients might be multifactorial, such as hepatitis B virus (HCV) and hepatitis B virus (HBV) coinfection and the side effects of antiretroviral therapy, a number of reports have documented that histological liver abnormalities occurred solely as a result of HIV-1 infection [13,18,19]. Nonetheless, few attempts have been made to elucidate the mechanisms of the liver damage in HIV-1-infected individuals.

In this study, we successfully isolated a CD4-independent clinical HIV-1 strain, designated SDA-1, from the viral quasispecies of a therapy-naive HIV-1 and *Pneumocystis jirovecii* pneumonia (PJP) patient in the late stage of AIDS with extremely low CD4 cell numbers. We characterized the phenotype of this virus and further explored whether it could infect or induce pathological effects in human hepatocytes.

Materials and methods

Patient's information

A 53-year-old Japanese man infected with HIV-1 was admitted to Tohoku University Hospital owing to prolonged fever and severe dyspnea in 2000. His plasma viral load and CD4 cell count at the time of admission was 220 000 copies/ml and 1 cell/μl, respectively. He was diagnosed with PJP, and his clinical stage was classified as IV-C3 [20]. The onset and route of HIV-1 infection were unknown. No evidence of coinfection with HBV or HCV in this patient was found. The patient was treated with trimethoprim and sulfamethoxazole (TMP–SMX) and highly active antiretroviral therapy (HAART). His condition deteriorated rapidly and he died 33 days after admission. Consent for autopsy was denied by the patient's family.

Before HAART, plasma samples and peripheral blood mononuclear cells (PBMC) were collected from this patient and cryopreserved in liquid nitrogen until use. The institutional Ethics Committee approved this study and written informed consent was obtained from the patient.

Virus isolation

HIV-1 isolation was achieved by using an in-vitro short-term phytohemagglutinin (PHA)–PBMC coculture method. Briefly, cryopreserved PBMC (2×10^6) from the patient were cocultivated with PHA-stimulated PBMC (5×10^6) from an HIV-1 seronegative healthy donor. The culture was maintained in RPMI-1640 (Invitrogen, California, USA) containing 10% fetal calf serum and 5 U/ml of recombinant interleukin-2 (IL-2) (Sigma, St. Louis, Missouri, USA). Proliferation of HIV-1 was examined by measuring p24 antigen in the cell culture supernatant using a p24 ELISA kit (RETRO-TEK, ZeptoMetrix Corp., New York, USA). The virus stocks were kept at −80 °C until use.

Amplification of *env* and sequence analysis

The full-length HIV-1 *env* genes were amplified by limiting dilution nested PCR from proviral PBMC DNA or plasma RNA as previously described [21,22]. To avoid artificial recombination and resampling of the viral genomes, independent nested PCR reactions were carried out per specimen [23,24].

The first round PCR was conducted with a F5852–R8935 primer pair (F5852, 5′-TAGAGCCCTGGAAG CATCCAGGAAG, HIV-1 HXB2 nucleotide position 5852–5876; R8935, 5′-TTGCTACTTGTGATTGCT CCATGT, HXB2 nucleotide position 8912–8935). The second round PCR was performed with a F5957–R8903 primer pair (F5957, 5′-GATCGAATTCTAGGCATC TCCTATGGCAGGAAGAAG, HXB2 nucleotide position 5957–5982, containing an additional *Eco*RI site (underlined) to facilitate cloning; R8903, 5′-AGCT CTC GAGGTCTCGAGATACTGCTCCCACCC, HXB2 nucleotide position 8881–8903, containing an additional *Xho*I site (underlined)). The purified PCR products were subcloned into the *Eco*RI and *Xho*I sites of the pSM-HXB2 plasmid. All correctly oriented *env* clones were then screened for biological function [22] followed by sequencing and phylogenetic analysis as previously described [25,26].

Cell lines and cell culture

All the cell lines, unless otherwise specifically mentioned, were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% fetal calf serum. Human glioma NP-2-CD4$^+$ cells transfected with a variety of chemokine receptors as indicated [27] were maintained in medium containing 500 μg/ml of G-418 (Promega, Wisconsin, USA) and 1 μg/ml of puromycin (Sigma). Human CD4-negative osteosarcoma (HOS) cells expressing either CXCR4 or CCR5 [28] were cultured in medium containing 1 μg/ml of puromycin. Human hepatoma cells Huh-7 and Hep-G2 [29] were obtained through the Cell Resource Center for Biomedical Research, Tohoku University, Japan. Human primary cultured hepatocytes (p-hepatocytes, BD Bioscience, California, USA) were maintained on BD Matrigel with Hepato-STIM hepatocyte culture medium (BD Bioscience).

Reagents and antibodies

The CXCR4 antagonist AMD3100 [30], and the CCR5 antagonist TAK-779 [31] were provided by the NIH AIDS Research and Reference Reagent Programme and Takeda Chemical Industries, Ltd., Osaka, Japan, respectively. Recombinant human soluble CD4 (sCD4) was from ImmunoDiagnostics, Inc. (Woburn, Massachusetts, USA). Antialbumin-fluorescein isothiocyanate (FITC) antibody was from Cedarlane Laboratories Ltd. (Hornby, Ontario, Canada). Anticytokeratin-18–phycoerythrin and antialpha fetoprotein (AFP)–FITC antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, California, USA). Anti-HIV-1-p24 (clone KC57)-FITC antibody was from Beckman Coulter. All other antibodies were from BD Pharmingen (San Diego, California, USA).

Pseudotyped virus infection assay

The HIV-1 Env-pseudotypes were generated as previously described [32]. Briefly, 293T cells (5×10^6 cells/10 cm-dish) were transfected with 5 μg of luciferase-expressing pNL4-3-Luc-R$^-$E$^-$ [33] or green fluorescent protein (GFP)-expressing pNL4-3-GFP [34] plasmid in combination with 10 μg of one of the *env*-expressing plasmids, pSM-SDA-1, pSM-HXB2 (X4), pSM-ADA (R5), or pSM-89.6 (R5X4). The vesicular stomatitis virus-G pseudotypes were also prepared [35].

For infection assays of luciferase-pseudotypes (luc-p), 10 ng p24 of luc-p were added into each well of 24-well plates (5×10^4 cells/well). After 12 h infection, the cells were washed and incubated for an additional 36 h at 37 °C. The cells were then lysed using a Luciferase Assay kit (Promega) and the luciferase activity was examined by a luminometer (Lumat 9507, Germany). To determine the effects of various reagents related to the viral receptors, target cells were preexposed for 1 h with the indicated concentration of the antagonists, or the antibodies. For GFP-pseudotypes (GFP-p) infection, target cells were infected with 10 ng p24 of GFP-p virus for 48 h and fixed by 5% paraformaldehyde. Infectivities were visualized under a Zeiss LSM510 confocal microscopy and DIC images with a 512 × 512 resolution were acquired.

Chimeric viruses

All *env* recombinant chimeric viruses in this study were generated in the background of pNL43, an X4-tropic HIV-1 infectious clone [36]. Briefly, the fragment of pNL43 containing *Eco*RI (nt 5743–5748) and *Kpn*I (nt 6343–6348) was amplified by PCR with a F5671–R6472 primer pair (F5671, 5′-GGCTCCATAACTTAGGA CAAC, pNL43 nucleotide position 5671–5691; R6472, 5′-TACTTCTTGTGGGTTGGGGTC, pNL43 position 6452–6472), followed by insertion into the pSM–SDA-1 using *Eco*RI and *Kpn*I. The new *Eco*RI–*Xho*I fragment (3155 bp) covering the entire SDA-1 *env* gene was then replaced with the equivalent region of pNL43 to construct the Env-chimeric virus NL43_SDA-1. Similarly, Env-chimeras of ADA (NL43_ADA), 89.6 (NL43_89.6) or truncated *env* (NL43_env (-)) were created, respectively. All Env-chimeric viruses were prepared by transfecting 293T cells as described above. For infection assays, 100 ng p24 of the chimeric viruses or virus stock supernatants were added in each well of 24-well plates (5×10^4 cells/well). After 2 h adsorption, the cells were washed and incubated for 48 h. Viral replication was monitored by p24 antigen production.

Flow cytometry and apoptosis assay

We performed cell-surface staining for CD4, CXCR4 and CCR5 by flow cytometry. To determine the purification and differentiation of p-hepatocytes, we tested the specific markers using antialbumin–FITC, anti-AFP–FITC and anticytokeratin-18–phycoerythrin antibodies. Appropriate class matched antibodies were used in each experiment. To detect the proliferation and intracellular p24, p-hepatocytes were fixed and permeabilized using a Cytofix-Cytoperm kit (BD Bioscience). Subsequently, the cells were stained with anti-Ki-67–phycoerythrin and antip24–FITC antibodies. Apoptosis of the p-hepatocytes was determined using the Apoptosis Detection kit I (BD Pharmingen). Flow cytometry analysis was performed using FACSCalibur (Becton Dickinson, New Jersey, USA). All Data were acquired and analyzed using Cell Quest software (BD Bioscience).

Nucleotide sequence accession number

The GenBank accession number for the sequence determined in this study is AY902478 (SDA-1).

Results

Evaluation of SDA-1 viral quasispecies

In an attempt to isolate CD4-independent clinical HIV-1 strain(s), we performed virus isolation from a

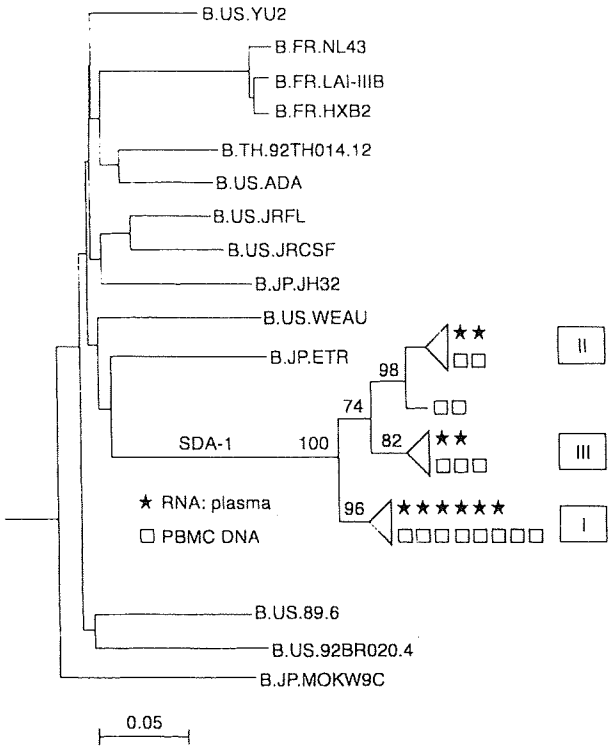

Fig. 1. Evolution of SDA-1 *env* quasispecies in plasma and PBMC. Phylogenetic analysis of newly characterized, SDA-1 gp120 *env* nucleotide sequences obtained from plasma ($n=10$) and PBMC ($n=15$) with representative sequences of HIV-1 subtype B. Numbers at branch nodes refer to the percentage of bootstrap values and symbols indicate individual clones.

therapy-naive HIV-1 and PJP patient with extremely low CD4 cell number, and successfully isolated the virus (peak of p24, 500 ng) from this patient and designated it SDA-1. To assess the quasispecies diversity present in *vivo*, we analyzed the SDA-1 *env* clones derived from plasma RNA and PBMC. As shown in Fig. 1, SDA-1 is grouped within the HIV-1 subtype B reference sequences. Within SDA-1's sequence cluster, three phylogenetic forms were identified. Supported by a significant bootstrap value (96%), form I was the predominant quasispecies, representing 70% of all sequences. Two minor quasispecies (forms II and III) had similar structures but differed in the position of the first breakpoint. The mean distances between major and minor quasispecies did not differ significantly from the sequence heterogeneity. Furthermore, the quasispecies diversities between plasma and PBMC were similar within each form, and were all below 5.0%.

Multicoreceptor usage and CD4-independent entry of SDA-1

To determine the receptor usage of SDA-1, we randomly selected five clones from the predominant quasispecies and generated Env-pseudotypes and Env-chimeric viruses as representatives. As a control, the Envs from a variety of HIV-1 subtypes with X4 (HXB2), R5 (ADA), and R5X4 (89.6) tropism were used. Utilizing luciferase-pseudotypes (luc-p), we first examined the coreceptor usage of SDA-1. We found that in the presence of CD4, all representative SDA-1 Env-pseudotypes were able to use efficiently both CXCR4 and CCR5, with additional moderate usage of CCR3 and CCR8 (Fig. 2a).

We next investigated whether SDA-1 Envs are capable of inducing CD4-independent infection. We found that SDA-1 Envs mediated entry into both HOS–CXCR4 and HOS–CCR5. However, the infectivities of SDA-1 for HOS–CXCR4 were approximately 2.5-fold higher than that for HOS–CCR5 (Fig. 2b). In stark contrast, none of the other types of luc-p viruses entered either of those cells. Furthermore, we evaluated the ability of SDA-1 Envs in mediating cell–cell fusion, a dye-transfer cell–cell fusion assay [37] was used with HOS–CXCR4 and HOS–CCR5 cells. Only in the cells expressing SDA-1 Envs (effector cells) did cell–cell fusion with CD4-negative, CXCR4- or CCR5-positive HOS cells (target cells) occur (data not shown).

In addition to the results with HOS–CXCR4 and CCR5, preexposure of HOS cells to Leu-3A, a CD4 monoclonal antibody (mAb) that recognizes the gp120 binding site on CD4 [38], failed to block SDA-1 infection. In contrast, pretreatment with antagonists for CXCR4 or CCR5 effectively inhibited infection (Table 1). Furthermore, the infectivities of SDA-1 on HOS–CXCR4 and HOS–CCR5 were enhanced by preexposure of the virus to sCD4 indicating that the binding of SDA-1 Env to CD4 induces further conformational changes in gp120 to fully expose the chemokine receptor binding domain. Collectively, SDA-1 Envs mediated the CD4-independent infection via both CXCR4 and CCR5.

Having clarified that SDA-1 is a CD4-independent isolate, we next investigated what types of $CD4^-$ cells are able to support SDA-1's entry. We focused first on human liver-derived cell lines, as the mechanisms of the liver damage in HIV-1-infected individuals are still unclear.

Two hepatoma cell lines, Huh-7 and Hep-G2, were used as targets. We first examined the expression of the receptors on the cell surface by flow cytometry and found that both CXCR4 and CCR5 were expressed on Huh-7 and Hep-G2 cells. In contrast, CD4 was not detected on either, which was confirmed by RT–PCR (data not shown). We then evaluated whether SDA-1 can enter into hepatoma cells with luc-p viruses. We found that only SDA-1 luc-p viruses efficiently infected Huh-7; however, its infectivity was marginal in Hep-G2 (Fig. 2c). Previous studies have shown that few HIV-1 variants can infect $CD8^+$ cells using CD8 as receptor [10,39]. Therefore, we further explored receptors used by

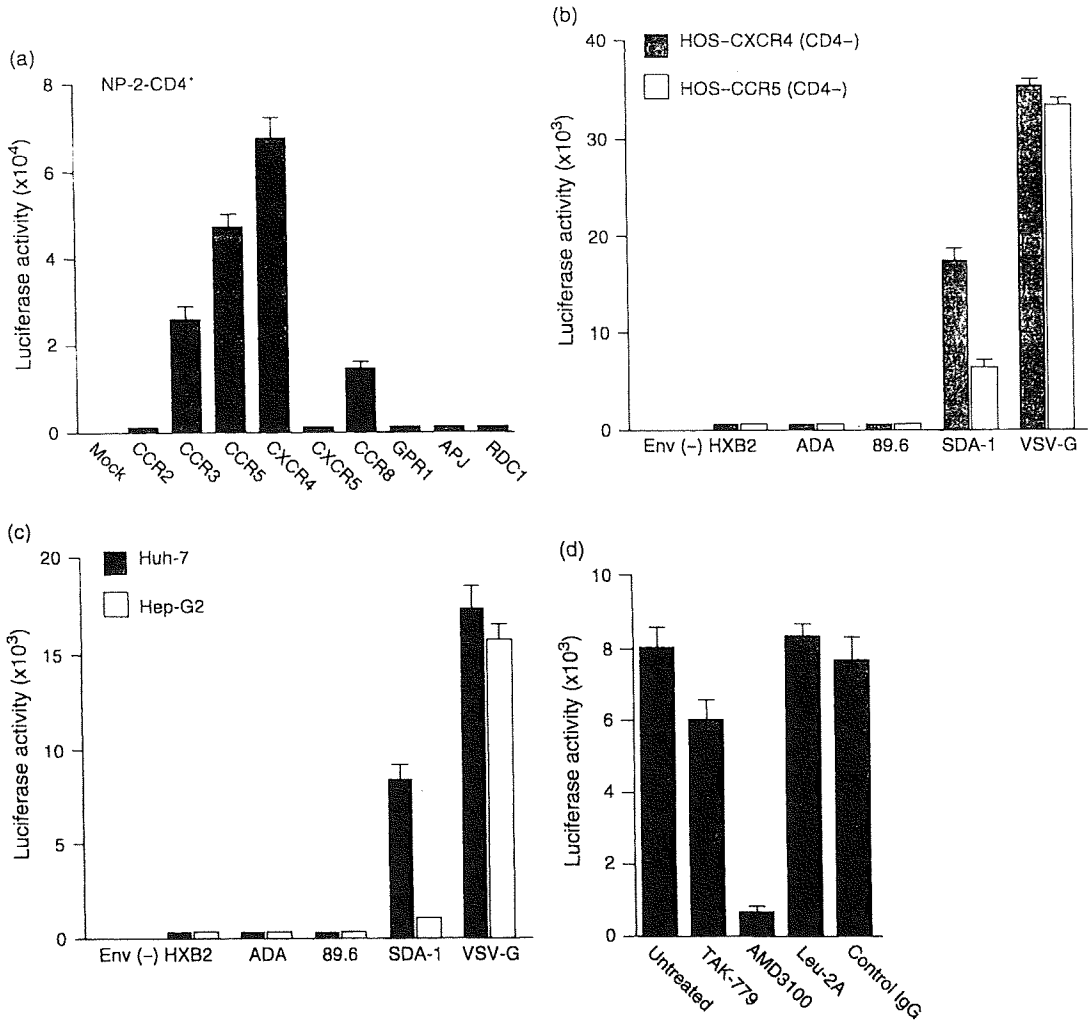

Fig. 2. Multicoreceptor usage and CD4-independent entry of SDA-1. (a) SDA-1 Envs mediate entry of $CD4^+$ cells using multiple coreceptors. NP-2-$CD4^+$ cells coexpressing one of the indicated chemokine receptors were exposed to SDA-1 luc-p viruses for 48 h and the luciferase activities were measured. (b) SDA-1 Envs mediate entry of $CD4^-$ cell lines through either CXCR4 or CCR5. The HOS cells ($CD4^-$) expressing either CXCR4 or CCR5 were exposed to the indicated HIV-1 luc-p viruses or VSV-G for 48 h, after which the infectivities were determined. (c) Entry of SDA-1 into $CD4^-$ human hepatoma cells. Huh-7 and Hep-G2 were exposed to the indicated HIV-1 luc-p viruses or VSV-G. Infectivities were determined at 48 h. (d) Effects of receptor-related antagonists or antibodies on the entry of SDA-1 entry into Huh-7 cells. Interaction of SDA-1 luc-p viruses with Huh-7 cells was tested in the absence or presence of AMD3100 (1.0 μM), TAK-779 (100 nM), anti-CD8 Leu-2A antibody (30 μg/ml) or class-matched control antibody (30 μg/ml). Results shown (a–d) are means of triplicate experiments. Bars, standard deviation. IgG, immunoglobulin G; VSV, vesicular stomatitis virus.

SDA-1 for entry into hepatoma cells. As shown in Fig. 2d, preexposure of Huh-7 to anti-CD8 Leu-2A mAb, as well as the CCR5 antagonist, TAK-779, failed to block SDA-1 infection of Huh-7, whereas anti-CXCR4 with AMD3100 effectively suppressed the infectivity. These results suggested that SDA-1 enters Huh-7 cells principally via CXCR4.

Replication of SDA-1 in human hepatoma cells

Although SDA-1 luc-p viruses infected some cells independently of CD4 cells, it was necessary to determine whether SDA-1 can replicate in those $CD4^-$ cells, particularly in hepatoma cells. For this purpose, we constructed NL43-based Env-chimeric viruses described above. We then examined whether the chimeric viruses were able to replicate in $CD4^-$ cells. As shown in Fig. 3a, the SDA-1 Env-chimeric viruses replicated efficiently in HOS–CXCR4 and HOS–CCR5 cells to similar levels. In contrast, none of the other Env-chimeric viruses infected either of those cells lines. Furthermore, we examined whether SDA-1 Env-chimeric viruses could replicate in hepatoma cells. As shown in Fig. 3b, high levels of NL43-SDA-1 replication were observed in Huh-7 cells. However, marginal replication was detected

Table 1. Inhibition of SDA-1 by blocking reagents in CD4⁻ cells.

Reagent	% Inhibition	
	HOS-CXCR4	HOS-CCR5
Medium	0	0
Control mAb (30 μg/ml)	0	0
Leu-3A (30 μg/ml)	10	12
Soluble CD4 (10 μg/ml)	225[a]	120[a]
AMD3100 (1.0 μM)	99	0
TAK-779 (100 nM)	0	97

CCR5, chemokine (C-C motif) receptor 5; CXCR4, chemokine (C-X-C motif) receptor 4; HOS, Human CD4-negative osteosarcoma; mAb, monoclonal antibody.
[a]Enhancement of entry.

in Hep-G2 cells. Although both Huh-7 and Hep-G2 cells are derived from human hepatoma, many potential host factors [40] could influence HIV replication, which for the most part remain unknown. Similarly, only Huh-7 cells, but not Hep-G2 cells, were susceptible to HCV [41,42]. These reasons may be related to the difference between Huh-7 and Hep-G2 regarding the level of replication by SDA-1.

SDA-1 replicates in both proliferating and static hepatocytes

To investigate further whether normal human hepatocytes could sustain entry and replication of SDA-1, p-hepatocytes were used for the following experiments. Among the three specific markers of human hepatocytes, both albumin and cytokeratin-18, but not alpha-fetoprotein were detected in the p-hepatocytes suggesting that the hepatocytes we used were well differentiated (data not shown). We also found that CXCR4 was expressed on the surface of p-hepatocytes. In contrast, neither CD4 nor CCR5 was detected on the p-hepatocyte surface or by real-time PCR (RT–PCR) (data not shown).

We next explored whether SDA-1 can enter p-hepatocytes by using GFP-p. As shown in Fig. 4a, only SDA-1 GFP-p viruses gave GFP-positive cells in p-hepatocytes, whereas other HIV-1 GFP-p viruses did not. The GFP-positive cells showed spindle-like shapes suggesting that the infection occurred in the p-hepatocytes but not in the contaminating lymphocytes. Furthermore, we studied whether SDA-1 can replicate in the p-hepatocytes. As shown in Fig. 4b, the p-hepatocytes were productively infected by the SDA-1 Env-chimeric viruses and SDA-1 virus stock itself but not by the other HIV-1 Env-chimeric viruses. Moreover, we found that AMD3100 inhibited the replication of SDA-1 in p-hepatocytes in a dose-dependent manner (Fig. 4c) indicating that the infection of p-hepatocytes by SDA-1 was mediated through CXCR4.

A previous study [19] reported that the HIV-1 gp120 *env* directly caused hepatocyte death by signaling through CXCR4 *in vitro*; however, most studies were performed using the hepatoma Huh-7 cells not hepatocytes, therefore, it may not really reflect the nature of liver damage. To explore the pathological effects of HIV-1 CD4-independent infection on hepatocytes, we exposed p-hepatocytes to the SDA-1 and analyzed cell viability. We found that the viability of the p-hepatocytes in cells cultured with or without SDA-1 Env-chimeric viruses was comparable (96%, P was not significant) indicating that HIV-1 CD4-independent infection rarely induces hepatocyte death via an apoptotic process (data not shown). To further examine whether the infection or replication of SDA-1 is limited only to a certain number of p-hepatocytes or whether the infectivity or replication is influenced by the cell cycle, we studied the intracellular expression by flow cytometry of p24 and Ki-67 [43], a marker strictly associated with cell proliferation, in the HIV-1-infected p-hepatocytes. As shown in Fig. 4d, we found that 32.49% of p-hepatocytes were infected by SDA-1. However, there was no significant difference in

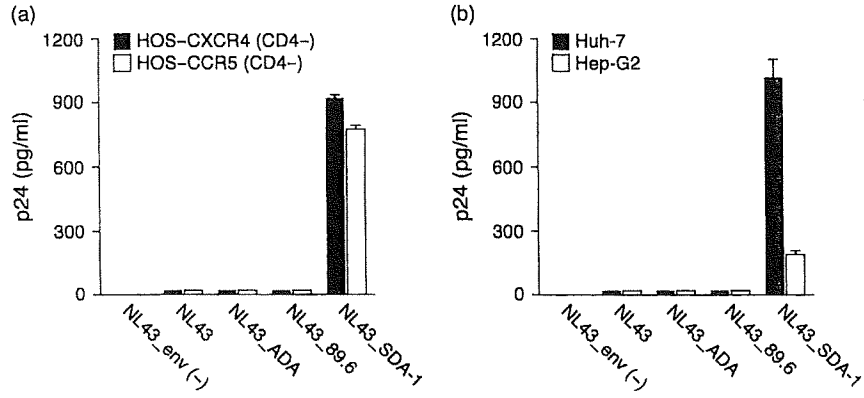

Fig. 3. CD4-independent infection of SDA-1 Env-chimeric viruses. The HOS cells (CD4⁻) expressing either CXCR4 or CCR5 (a) and two CD4⁻ human hepatoma cells (b) were incubated with the indicated HIV-1 Env-chimeric viruses. Virus replication was then monitored by p24 antigen production on day 3. Results shown (a, b) are means of triplicate experiments. Bars, standard deviation.

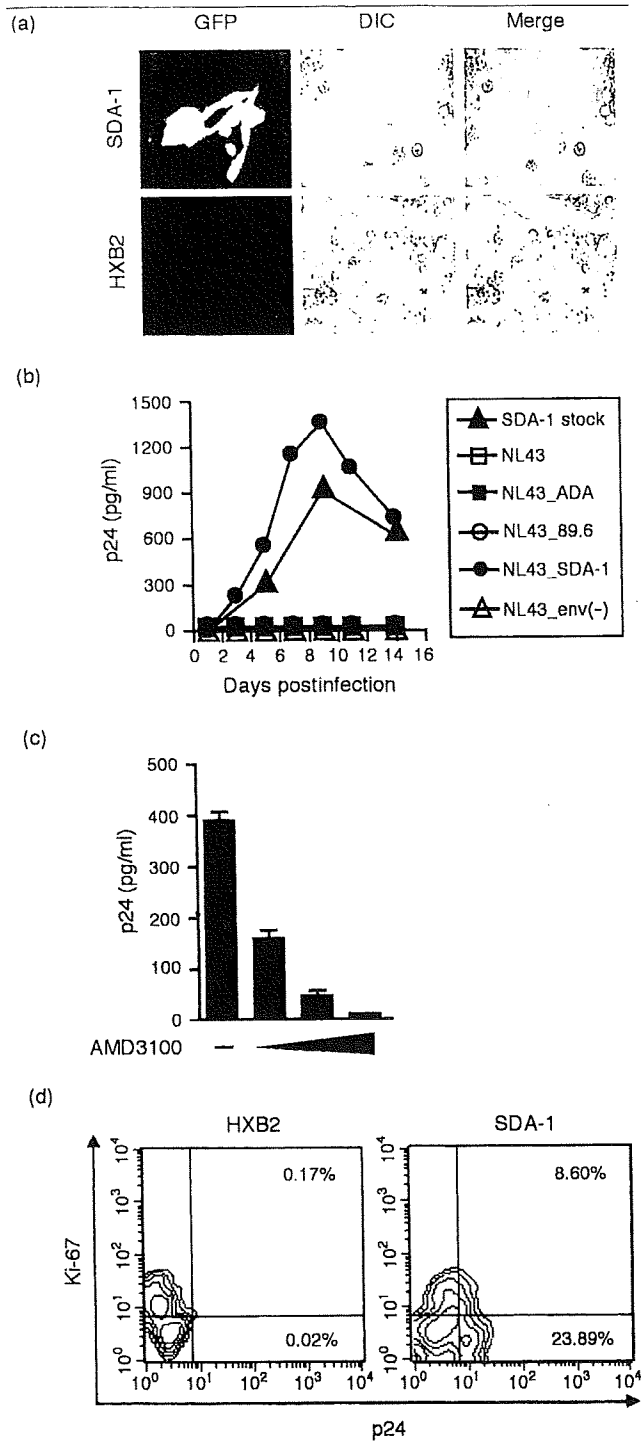

Fig. 4. SDA-1 enters and replicates in CD4$^-$ human p-hepatocytes. (a) Entry of SDA-1 into p-hepatocytes. The p-hepatocytes were exposed to the indicated HIV-1 GFP-p viruses for 48 h. Infectivity was determined as GFP$^+$ cells by confocal microscopy. (b) Replication of SDA-1 Env-chimeric viruses and SDA-1 virus stock in human p-hepatocytes. (c) SDA-1 infects p-hepatocytes through CXCR4. The inhibitory effects of AMD 3100 (0.1, 0.3 and 1.0 μM) on SDA-1 Env-chimeric viruses infection of p-hepatocytes were studied. Results shown are means of triplicate experiments. Bars, SD. (d) SDA-1 replicates in both proliferating and static human p-hepatocytes. Intracellular stainings of HIV-1-infected p-hepatocytes for p24 and Ki-67 were analyzed by flow cytometry. CXCR4, chemokine (C-X-C motif) receptor 4; GFP-p, GFP-pseudotypes.

percentage of p24 expression between Ki-67$^+$ (31%) and Ki-67$^-$ p-hepatocytes (33.1%), suggesting that SDA-1 efficiently enters and replicates in both proliferating and static hepatocytes.

Considering that SDA-1 can infect hepatocytes *in vitro*, it would have been interesting to determine whether the patient's liver was infected *in vivo*. However, consent for a liver biopsy was denied by the patient's family. There was no evidence of liver dysfunction. When virus was isolated from this patient; however, liver damage [an aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio ≥ 1] was observed at the end of the clinical stage. Although the cause of liver injury was unclear, our present data suggest that CD4-independent HIV-1 infection may lead to hepatocellular damage.

Discussion

In this study, we characterized a quasispecies of a CD4-independent HIV-1 isolate, termed SDA-1, which was able to utilize either CXCR4 or CCR5 in the absence of CD4. Moreover, we demonstrated that SDA-1 efficiently entered and replicated in Huh-7 hepatoma cells and normal human hepatocytes, through CXCR4, without inducing apoptotic cell death.

Many SIV and HIV-2 isolates can infect cells without CD4, at least to some extent. However, CD4-independent HIV-1 viruses have been rarely isolated and, so far, only a few laboratory-adapted CD4-independent HIV-1 variants have been reported. It must be noted that CD4-independent HIV-1 variants, isolated *in vitro* by passage through cells lacking CD4, have been shown to be more sensitive to neutralizing antibodies than CD4-dependent viruses [44,45]. Therefore, we might hypothesize that the emergence of a quasispecies of HIV-1 with a reduced requirement for CD4 is likely to be at a low abundance relative to the more common CD4$^+$ strains. However, with disease progression, HIV-1 variants with reduced affinity for CD4 and with increased affinity for chemokine receptor could evolve and become more robust in the viral quasispecies, disseminate in a variety of CD4$^-$ tissues *in vivo* under conditions of both reduced immunological pressure and a dramatically reduced pool of target CD4$^+$ cells concomitant with high levels of virus replication. It will be important to search the viral quasispecies in other patients, especially in the later stages of HIV-1 disease for the existence of similar CD4-independent HIV-1 variants and expanded cellular tropism.

Although the extent to which CD4 cells are infected *in vivo* is unclear, it has been widely thought to be low. Nonetheless, recent studies [11,12] utilizing the novel approach of laser capture microscopy have revealed HIV-1 sequences in isolated CD4 cells of kidney epithelium and neuronal cells, indicating that latent infection might occur in such cells or tissues *in vivo*. The mechanism of viral entry into CD4 cells remains unclear, but as we show here the evidence of emergence of CD4-independent strains *in vivo* must be kept in mind.

End-stage liver disease is now becoming a frequent cause of death in HIV-1-infected hospitalized patients. HCV and HBV coinfection with HIV-1 has been shown to enhance the progression of liver damage [16]. However, little attention has been given to the direct virological interaction between HIV and HCV/HBV in the liver, as HIV has been thought not to infect hepatocytes directly. Nonetheless, a number of reports have documented that histological liver abnormalities occurred solely as a result of HIV-1 infection. In our study, we clearly demonstrated that SDA-1 efficiently enters and replicates in both proliferating and static hepatocytes through CXCR4. To our knowledge, this is the first report that HIV-1 can efficiently replicate in normal hepatocytes. Furthermore, we have shown that HIV-1 infection did not induce significant cytocidal effects in the hepatocytes. It is noteworthy that the liver is a continuously regenerating organ. Therefore, if HIV-1 enters and integrates its DNA into the host genome, liver cells containing HIV-1 DNA will be continuously generated by the division of the infected cells. Thus, the expression of HIV-1 proteins on the infected cell surface might result in chronic damage of the liver cells by inducing host immune responses. Direct virological interaction between HIV, HCV and HBV in the liver or enhanced production of HIV-1 by inflammatory cytokines produced by the HCV and HBV-activated immune cells might also exacerbate the liver injury. At present, however, we have no definite information concerning the extent to which patients' hepatocytes harbor HIV-1 and CD4-independent HIV-1 variants.

Finally, a particularly important area of vaccine research is to take advantage of gp120 structural information to guide the design of novel envelope immunogens. As has been reported, CD4-dependent viruses hide neutralizing epitopes and only CD4 binding to gp120 induces conformational changes in gp120 to fully expose epitopes for broadly neutralizing antibodies. The CD4-independent strain we isolated here seems particularly important, as it can efficiently replicate in $CD4^-$ hepatocytes. Therefore, the gp120 structural alterations, which might expose the coreceptor binding site without binding to CD4, may also open up other sites that could yield neutralizing antibodies. Nevertheless, evidence of a clinical CD4-independent R5X4 HIV-1 virus should have important implications concerning the range of mutability and tropism of HIV-1 and the pathogenesis of AIDS.

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P.X., H.L., Y.S. and T.H. designed the study. P.X., O.U., Y.S., M.Z., Y.A. and H.G. performed the experiments. P.X., O.U, Y.S., H.L. and T.H. analyzed the data. N.S. and H.H. contributed to the coreceptor expressing cell lines. P.X., H.L., Y.S., O.U., N.S., H.H. and T.H. contributed to writing the paper. T.H. contributed to grant application and financial support.

References

1. Dalgleish AG, Beverley PC, Clapham PR, Crawford DH, Greaves MF, Weiss RA. **The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus.** *Nature* 1984; **312**:763–767.
2. Feng Y, Broder CC, Kennedy PE, Berger EA. **HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor.** *Science* 1996; **272**:872–877.
3. Berger EA, Doms RW, Fenyo EM, Korber BT, Littman DR, Moore JP, *et al.* **A new classification for HIV-1.** *Nature* 1998; **391**:240.
4. Berger EA, Murphy PM, Farber JM. **Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease.** *Annu Rev Immunol* 1999; **17**:657–700.
5. Edinger AL, Mankowski JL, Doranz BJ, Margulies BJ, Lee B, Rucker J, *et al.* **CD4-independent, CCR5-dependent infection of brain capillary endothelial cells by a neurovirulent simian immunodeficiency virus strain.** *Proc Natl Acad Sci USA* 1997; **94**:14742–14747.
6. Reeves JD, Hibbitts S, Simmons G, McKnight A, Azevedo-Pereira JM, Moniz-Pereira J, Clapham PR. **Primary human immunodeficiency virus type 2 (HIV-2) isolates infect CD4-negative cells via CCR5 and CXCR4: comparison with HIV-1 and simian immunodeficiency virus and relevance to cell tropism in vivo.** *J Virol* 1999; **73**:7795–7804.

7. Dumonceaux J, Nisole S, Chanel C, Quivet L, Amara A, Baleux F, *et al.* **Spontaneous mutations in the env gene of the human immunodeficiency virus type 1 NDK isolate are associated with a CD4-independent entry phenotype.** *J Virol* 1998; **72**:512–519.
8. Hoffman TL, LaBranche CC, Zhang W, Canziani G, Robinson J, Chaiken I, *et al.* **Stable exposure of the coreceptor-binding site in a CD4-independent HIV-1 envelope protein.** *Proc Natl Acad Sci USA* 1999; **96**:6359–6364.
9. Kolchinsky P, Mirzabekov T, Farzan M, Kiprilov E, Cayabyab M, Mooney LJ, *et al.* **Adaptation of a CCR5-using, primary human immunodeficiency virus type 1 isolate for CD4-independent replication.** *J Virol* 1999; **73**:8120–8126.
10. Zerhouni B, Nelson JA, Saha K. **Isolation of CD4-independent primary human immunodeficiency virus type 1 isolates that are syncytium inducing and acutely cytopathic for CD8+ lymphocytes.** *J Virol* 2004; **78**:1243–1255.
11. Torres-Munoz J, Stockton P, Tacoronte N, Roberts B, Maronpot RR, Petito CK. **Detection of HIV-1 gene sequences in hippocampal neurons isolated from postmortem AIDS brains by laser capture microdissection.** *J Neuropathol Exp Neurol* 2001; **60**:885–892.
12. Marras D, Bruggeman LA, Gao F, Tanji N, Mansukhani MM, Cara A, *et al.* **Replication and compartmentalization of HIV-1 in kidney epithelium of patients with HIV-associated nephropathy.** *Nat Med* 2002; **8**:522–526.
13. Cao YZ, Dieterich D, Thomas PA, Huang YX, Mirabile M, Ho DD. **Identification and quantitation of HIV-1 in the liver of patients with AIDS.** *AIDS* 1992; **6**:65–70.
14. Housset C, Lamas E, Brechot C. **Detection of HIV1 RNA and p24 antigen in HIV1-infected human liver.** *Res Virol* 1990; **141**:153–159.
15. Bica I, McGovern B, Dhar R, Stone D, McGowan K, Scheib R, Snydman DR. **Increasing mortality due to end-stage liver disease in patients with human immunodeficiency virus infection.** *Clin Infect Dis* 2001; **32**:492–497.
16. Martin-Carbonero L, Soriano V, Valencia E, Garcia-Samaniego J, Lopez M, Gonzalez-Lahoz J. **Increasing impact of chronic viral hepatitis on hospital admissions and mortality among HIV-infected patients.** *AIDS Res Hum Retroviruses* 2001; **17**:1467–1471.
17. Sabin CA. **The changing clinical epidemiology of AIDS in the highly active antiretroviral therapy era.** *AIDS* 2002; **16 Suppl 4**:S61–S68.
18. Lefkowitch JH. **Pathology of AIDS-related liver disease.** *Dig Dis* 1994; **12**:321–330.
19. Vlahakis SR, Villasis-Keever A, Gomez TS, Bren GD, Paya CV. **Human immunodeficiency virus-induced apoptosis of human hepatocytes via CXCR4.** *J Infect Dis* 2003; **188**:1455–1460.
20. **Centers for Disease Control and Prevention: 1993 revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults.** [No. RR-17] *MMWR Morb Mortal Wkly Rep* 1992; **41**:1–19.
21. Wei X, Decker JM, Wang S, Hui H, Kappes JC, Wu X, *et al.* **Antibody neutralization and escape by HIV-1.** *Nature* 2003; **422**:307–312.
22. Derdeyn CA, Decker JM, Bibollet-Ruche F, Mokili JL, Muldoon M, Denham SA, *et al.* **Envelope-constrained neutralization-sensitive HIV-1 after heterosexual transmission.** *Science* 2004; **303**:2019–2022.
23. Liu SL, Rodrigo AG, Shankarappa R, Learn GH, Hsu L, Davidov O, *et al.* **HIV quasispecies and resampling.** *Science* 1996; **273**:415–416.
24. Shankarappa R, Margolick JB, Gange SJ, Rodrigo AG, Upchurch D, Farzadegan H, *et al.* **Consistent viral evolutionary changes associated with the progression of human immunodeficiency virus type 1 infection.** *J Virol* 1999; **73**:10489–10502.
25. Thompson JD, Higgins DG, Gibson TJ. **CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice.** *Nucl Acids Res* 1994; **22**:4673–4680.
26. Gerhardt M, Mloka D, Tovanabutra S, Sanders-Buell E, Hoffmann O, Maboko L, *et al.* **In-depth, longitudinal analysis of viral quasispecies from an individual triply infected with late-stage human immunodeficiency virus type 1, using a multiple PCR primer approach.** *J Virol* 2005; **79**:8249–8261.
27. Liu HY, Soda Y, Shimizu N, Haraguchi Y, Jinno A, Takeuchi Y, Hoshino H. **CD4-Dependent and CD4-independent utilization of coreceptors by human immunodeficiency viruses type 2 and simian immunodeficiency viruses.** *Virology* 2000; **278**:276–288.
28. Landau NR, Littman DR. **Packaging system for rapid production of murine leukemia virus vectors with variable tropism.** *J Virol* 1992; **66**:5110–5113.
29. Nakabayashi H, Taketa K, Miyano K, Yamane T, Sato J. **Growth of human hepatoma cells lines with differentiated functions in chemically defined medium.** *Cancer Res* 1982; **42**:3858–3863.
30. Hendrix CW, Flexner C, MacFarland RT, Giandomenico C, Fuchs EJ, Redpath E, *et al.* **Pharmacokinetics and safety of AMD-3100, a novel antagonist of the CXCR-4 chemokine receptor, in human volunteers.** *Antimicrob Agents Chemother* 2000; **44**:1667–1673.
31. Baba M, Nishimura O, Kanzaki N, Okamoto M, Sawada H, Iizawa Y, *et al.* **A small-molecule, nonpeptide CCR5 antagonist with highly potent and selective anti-HIV-1 activity.** *Proc Natl Acad Sci USA* 1999; **96**:5698–5703.
32. He J, Chen Y, Farzan M, Choe H, Ohagen A, Gartner S, *et al.* **CCR3 and CCR5 are co-receptors for HIV-1 infection of microglia.** *Nature* 1997; **385**:645–649.
33. Connor RI, Chen BK, Choe S, Landau NR. **Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes.** *Virology* 1995; **206**:935–944.
34. Reiser J. **Production and concentration of pseudotyped HIV-1-based gene transfer vectors.** *Gene Ther* 2000; **7**:910–913.
35. Li SL, Zhang XY, Ling H, Ikeda J, Shirato K, Hattori T. **A VSV-G pseudotyped HIV vector mediates efficient transduction of human pulmonary artery smooth muscle cells.** *Microbiol Immunol* 2000; **44**:1019–1025.
36. Adachi A, Gendelman HE, Koenig S, Folks T, Willey R, Rabson A, Martin MA. **Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone.** *J Virol* 1986; **59**:284–291.
37. Furuta RA, Wild CT, Weng Y, Weiss CD. **Capture of an early fusion-active conformation of HIV-1 gp41.** *Nat Struct Biol* 1998; **5**:276–279.
38. Sattentau QJ, Dalgleish AG, Weiss RA, Beverley PC. **Epitopes of the CD4 antigen and HIV infection.** *Science* 1986; **234**:1120–1123.
39. Saha K, Zhang J, Gupta A, Dave R, Yimen M, Zerhouni B. **Isolation of primary HIV-1 that target CD8+ T lymphocytes using CD8 as a receptor.** *Nat Med* 2001; **7**:65–72.
40. Brass AL, Dykxhoorn DM, Benita Y, Yan N, Engelman A, Xavier RJ, *et al.* **Identification of host proteins required for HIV infection through a functional genomic screen.** *Science* 2008; **319**:921–926.
41. Cormier EG, Tsamis F, Kajumo F, Durso RJ, Gardner JP, Dragic T. **CD81 is an entry coreceptor for hepatitis C virus.** *Proc Natl Acad Sci USA* 2004; **101**:7270–7274.
42. Jopling CL, Yi M, Lancaster AM, Lemon SM, Sarnow P. **Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA.** *Science* 2005; **309**:1577–1581.
43. Harrison RF, Rowlands DC. **Proliferating cell nuclear antigen as a marker of hepatocyte proliferation.** *Lancet* 1994; **343**:1290–1291.
44. Edwards TG, Hoffman TL, Baribaud F, Wyss S, LaBranche CC, Romano J, *et al.* **Relationships between CD4 independence, neutralization sensitivity, and exposure of a CD4-induced epitope in a human immunodeficiency virus type 1 envelope protein.** *J Virol* 2001; **75**:5230–5239.
45. Kolchinsky P, Kiprilov E, Sodroski J. **Increased neutralization sensitivity of CD4-independent human immunodeficiency virus variants.** *J Virol* 2001; **75**:2041–2050.

HAARTによる脂質代謝異常と高分子アディポネクチンの関連

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ATVへの変更はHMW-Ad濃度を回復させる

メタボリックシンドロームにおける病態の一部は，成熟脂肪細胞から分泌されるアディポサイトカインによって説明できることが明らかになってきた。特に活性体である高分子量アディポネクチン(HMW-Ad)は，インスリン抵抗性改善作用，抗動脈硬化作用を有するため重要視されている。そこで，抗HIV薬の副作用として起こってくる代謝障害において，HMW-Adがどのように関与しているかを検討した。

当院通院中のHIV感染者57例を，HAARTのキードラッグ別にエファビレンツ(EFV)群，アタザナビル(ATV)以外のプロテアーゼ阻害薬(PI)群，ATV群，他剤からATVへの変更群に分け，HAART開始前，開始後もしくは薬剤変更後1年以内に血清脂質とHMW-Adを測定した。

EFV群とPI群では中性脂肪(TG)と低比重リポ蛋白コレステロール(LDL-C)が増加し，HMW-Ad濃度が低下したが，ATV群では有意な影響を認めなかった。また，他剤からATVへの変更群ではLDL-Cが低下，HMW-Ad濃度が上昇し，その差は有意であった(**表1**，**図1**)。リポジストロフィーを有する患者では，HMW-Adの変化率が大きい傾向にあり，両者の関連性が認められた(**図2**)。

抗HIV薬による脂肪細胞減少がHMW-Ad低下の一因

*In vitro*において，脂肪前駆細胞(3T3-L1)にイソブチルメチルキサンチン，デキサメタゾン，インスリンを添加して分化誘導し，分化前・分化後に抗HIV薬

表1　BMI，脂質の変化

	BMI 前	BMI 後	TG 前	TG 後	LDL-C 前	LDL-C 後	HDL-C 前	HDL-C 後
EFV	21.4±3.3	21.2±3.6	144.4±36.8	233.5±125.4**	108.9±35.4	122.0±46.2**	42.8±16.1	53.5±11.5**
PI	21.6±2.9	21.7±3.2	151.1±60.0	231.9±105.6*	95.7±41.3	128.5±40.3*	37.6±11.5	46.9±7.4**
ATV	22.3±3.0	22.8±3.4	165.1±104.8	174.4±85.4	88.5±16.1	104.3±23.2*	38.7±11.3	41.0±8.0
他剤→ATV	21.1±2.9	20.7±2.5	188.4±92.0	189.1±111.3	120.7±61.5	101.5±52.0**	54.6±28.9	55.1±52.0

*$p < 0.001$. **$p < 0.005$ (paired t 検定)

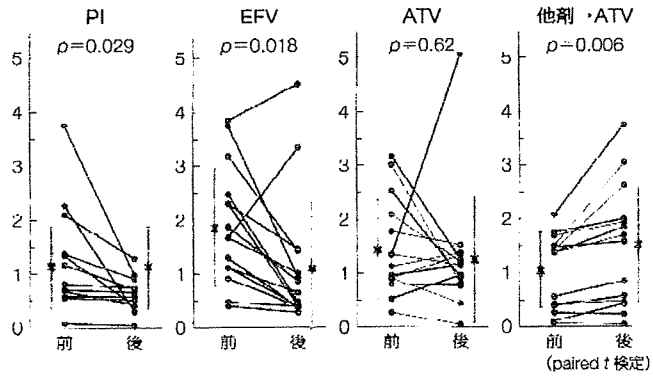

図1　HMW-Adの変化

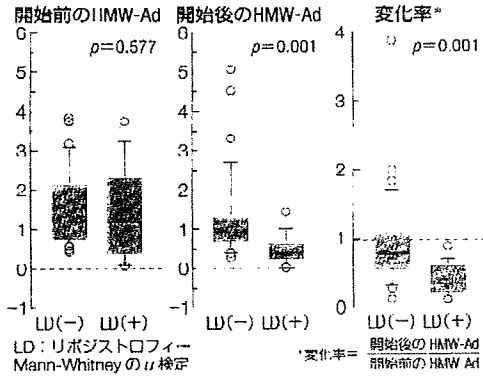

図2　リポジストロフィーとの関連

[ATV, リトナビル(RTV), ネルフィナビル(NFV), EFV を添加し, 脂肪細胞の分化, HMW-Ad 産生に与える影響を解析した. 脂肪前駆細胞, 成熟脂肪細胞に EFV, NFV を添加すると, 脂肪滴は著明に減少した(図3). 成熟脂肪細胞において, HMW-Ad 産生は RTV, NFV, EFV の添加により著明に抑制されたが, ATV 添加の影響は軽度であった. また NFV, EFV によって著明に抑制された HMW-Ad は, 薬剤を ATV に変更することで回復した(図4).

以上, 抗 HIV 薬の脂肪細胞への影響は薬剤によって異なるが, ATV では影響が少なかった. また, 成熟脂肪細胞の減少にともう HMW-Ad 低下が抗 HIV 薬における脂質代謝異常の一因と考えられた.

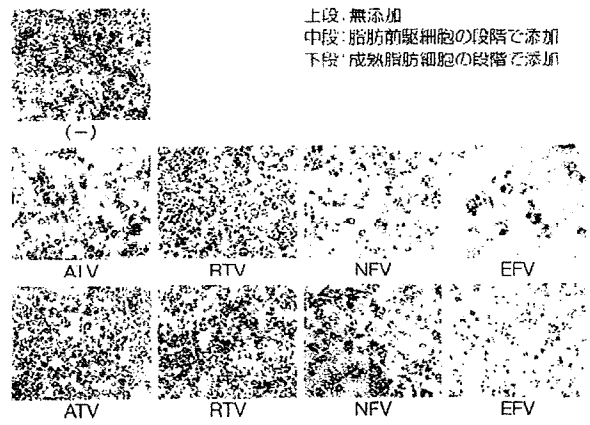

図3 313-L1の変化

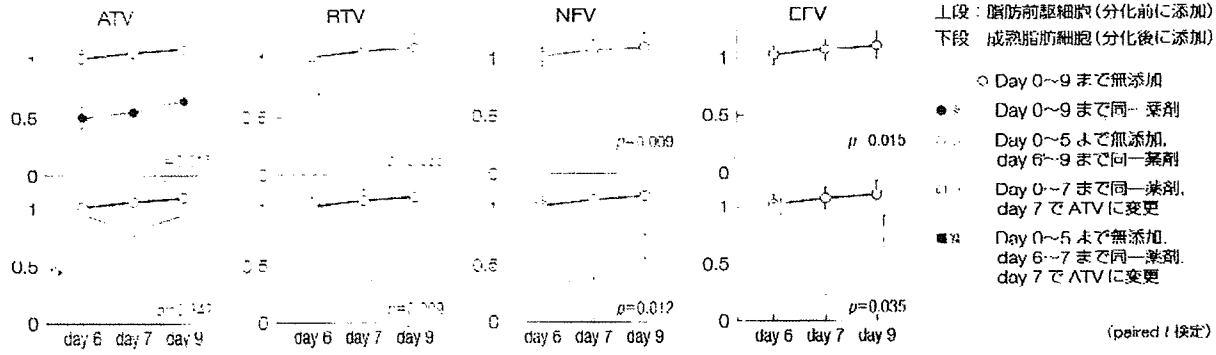

図4 アディポネクチンの産生(リアルタイム PCR)

原　著

HIV脳症5例の臨床的特徴と経過

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要旨：HIV脳症5症例を報告した．1996年から2005年11月の間に名古屋医療センターを受診したHIV感染症458症例（うちAIDSは127症例）を対象とした．HIV脳症と診断した症例はいずれも高度の免疫不全状態にあり，他の日和見感染症を3症例にみとめた．4症例はHIV感染症が判明したのとほぼ同時期にHIV脳症と診断された．5症例ともHIVに対して抗ウイルス療法は未施行であった．HAARTを施行することで全例で症状の改善をみとめ，死亡はみとめなかった．精神科介入を要したり1例を除いて社会復帰できないなど，行動障害を呈したHIV脳症の機能予後は不良であり，HAARTのみの治療効果は不十分と考えられた．

（臨床神経，48：173—178，2008）

Key words：HIV，AIDS，認知障害，行動異常，予後

はじめに

HIV感染症は病期が進行するにつれ日和見感染症など各種疾患を合併する．なかでもHIV脳症はAIDS指標疾患の1つであり，中枢神経領域における重要な合併症として挙げられる．亜急性から慢性に進行する記銘力低下，注意や意欲の低下，思考緩慢といった認知障害と，動作緩慢や失調性歩行などの運動障害を呈し，頭部MRI T_2強調画像やFLAIR画像にて大脳白質から基底核にかけてびまん性の高信号を生じ皮質下は保たれることを特徴とする[1)]．しかし我が国ではHIV脳症の臨床報告は非常に少ない．そこでわれわれは，HIV東海北陸ブロック拠点病院である当院で経験したHIV脳症の自験5症例について，その臨床的特徴と経過について検討した．

対　象

1996年から2005年11月に名古屋医療センター（以下当院）内科を受診したHIV感染症のうち，神経内科に紹介された症例のうちHIV脳症と診断されたものを対象とした．

方　法

当院内科より神経内科を紹介受診したHIV感染症の症例に対し，著者の神経内科医2名によって神経学的診察，髄液検査，頭部MRIを施行した．認知障害と運動障害の双方をみとめ，血液検査，髄液検査，各種画像検査にて代謝異常や日和見感染症，悪性腫瘍等が除外されたものをHIV脳症と診断し，神経学的所見，長谷川式簡易痴呆スケール（以下HDS-R）もしくはMini-mental State Examinaton（以下MMSE），CD4陽性細胞数（以下CD4）および血清HIVウイルス量，頭部MRIにて経過を追跡した．

結　果

上記期間に累計458症例のHIV患者が受診し，そのうちAIDS発症者は127症例であった．AIDSのうち25例に中枢神経合併症をみとめた．中枢神経合併症の内訳はTable 1にまとめた．HIV脳症は5例にみとめた．HIV感染症の感染経路は5例とも同性間性行為と推定された．全例でHAARTを施行し，CD4の改善とHIVウイルス量の抑制をみとめた．以下，症例を提示する．

症例1　37歳男性

職業はデザイン関係．2003年8月から微熱と歩行障害が出現し，同年9月に動けなくなり前医に入院した．10月に尿閉が出現．頭部MRIでは大脳，脳幹にびまん性病変があり，ADEMもしくはウェルニッケ脳症と診断され，ステロイドパルス療法とビタミンB大量を投与されるも効果なし．その後，HIV抗体陽性と判明したため，12月に当院に転院した．体温38.6℃，臥床状態で，四肢の関節腫脹があった．自発的に開眼し，寡動．発語は「イタイ」など限られた単語のみであった．知能はHDS-Rは1点（場所について「病院」を選択できた），WAIS-Rは判定不能．脳神経はほぼ正常であり，運動は指示にしたがえず評価不能，両側に強制把握をみとめた．上肢

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Table 1　Complications of HIV infection in central nervous system

	-July 2000	August 2000-April 2003	May 2003-November 2005
Toxoplasmic encephalitis	3	1	2
PML	2		1
Cryptoccocal meningitis	1	1	1
Primary CNS lymphoma	1	1	
HIV encephalopathy			5
Tuberculous meningitis			1
Cytomegaloviral encephalitis			1
Other		Unknown 1 Amebic encephalitis 1	Viral meningitis 1 Hydrocephalus 1
Total	7	5	13
HIV infectious cases (AIDS cases)	106 (19)	231 (56)	363 (127)
Rate of CNS complications with AIDS	36.8%	8.9%	9.4%

PML: Progressive multifocal leukoencephalopathy

Table 2　Counts of CD4+ lymphocytes and HIV viral load before and after HAART

	Case 1	2	3	4	5
CD4+ lymphocytes (before → after HAART) (/μl)	71 → 276	11 → 281	8 → 74	17 → 244	5 → 259
HIV viral load (before → after HAART) (copies/ml)	2.1×10^5 → < 50	5.3×10^5 → < 50	5.3×10^5 → < 50	1.2×10^7 → 1.2×10^3	1.3×10^6 → < 50

Table 3　Findings of cerebrospinal fluid at time of HIV encephalopathy diagnosis

	Case 1	2	3	4	5
Cell counts (/μl)	2	5	7	3	47
Protein (mg/dl)	45	46	50	25	21
Glucose (blood glucose) (mg/dl)	29 (80)	48 (100)	48 (126)	48 (96)	85 (244)
β-2 microglobulin (μg/ml)	7.1	—	—	3.5	3.7
HIV viral load (copies/ml)	5.9×10^4	—	1.7×10^4	1.8×10^3	9.3×10

—: not examined

に振戦があり，四肢に筋強剛をみとめた．腱反射は全体に減弱し，バビンスキー徴候は両側陽性，尿閉のため尿道カテーテルが留置されており便失禁状態であった．血液検査（Table 2）ではCD4 71/μl，HIVウイルス量2.1×10^5copies/ml，HBs抗原陽性であり，HCV抗体，梅毒，β-D-グルカン，サイトメガロウイルスC10/C11抗原，トキソプラズマIgM/IgG抗体，クリプトコッカス抗原はいずれも陰性であった．髄液検査（Table 3）では細胞数2/μl，蛋白45mg/dl，糖29mg/dl，HIVウイルス量5.9×10^4copies/ml，β-2ミクログロブリン7.1μg/ml．墨汁染色陰性，結核菌，非定型抗酸菌，サイトメガロウイルス，JCウイルスのPCRはすべて陰性であった．また，一般細菌，抗酸菌，真菌培養はいずれも陰性で，細胞診も陰性であった．脳波は6～7Hzの全般性徐波をみとめた．神経伝導速度では上肢は筋電図混入が強く判定不能で，下肢はF波をふくめ正常であった．入院時頭部MRI（Fig. 1）では，脳幹および大脳白質にびまん性に広がる高信号域をみとめた．

以上から，HIV脳症と診断し，発熱の原因はカテーテル留置にともなう尿路感染症と思われた．2004年1月よりHAARTを施行し，その約1カ月後より発動性と運動障害は改善したが，下肢関節は拘縮変形のため立位歩行はできなかった．6カ月後のHDS-R 17点，WAIS-Rは言語性IQ 88，動作性IQ 69，全体IQ 77．20カ月後にはHDS-R 22点と，認知機能障害は不完全ながらも徐々に改善傾向を示した．HAART開始1カ月後においてCD4 201/μl，HIVウイルス量2.3×10^3copies/mlと改善し，22カ月後ではCD4 226/μl，ウイルス量は検出感度以下とさらに改善した．しかし，人格変化がいちじるしく，周囲に対して攻撃的言動をとったり，夜間大声で叫ぶなどの精神症状が強かったために精神科介入による投薬をおこない，約1年後に施設入所となった．頭部MRIの経時変化をFLAIR画像（Fig. 1）にて検討すると，両側左右対称性の大脳前頭葉から基底核にかけて白質の萎縮が進行していた．

症例2　35歳男性．

27歳時に梅毒の既往がある．2004年6月，乾性咳嗽，労作