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Anti-human immunodeficiency virus (HIV) agents are also potent and selective inhibitors of feline immunodeficiency virus (FIV)-induced cytopathic effect: development of a new method for screening of anti-FIV substances in vitro

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Summary

The ability of several known anti-HIV substances to inhibit feline immunodeficiency virus (FIV) was tested. The results showed that FIV infection of feline T-cells was almost completely blocked in the presence of all of the agents tested. However, FIV-induced syncytium formation between a human T-cell line (MT-2 cells) and a FIV-infected feline lymphocyte cell line (3201/FIV) was inhibited only by dextran sulfate and pradimicin A. The assay used to measure syncytium inhibition was rapid and did not use potentially hazardous human immunodeficiency virus (HIV)-infected cells. The efficacy results coincided with those of HIV studies.

Feline immunodeficiency; Virus; Antiviral; Cell fusion; Inhibition

A feline lentivirus, Petaluma strain, was first isolated from the peripheral blood lymphocytes (PBL) of a cat with a chronic acquired immunodeficiency syndrome by Pedersen et al. (1987), molecularly characterized by Olmsted et al. (1989) and named feline immunodeficiency virus (FIV). FIV-infected cats appear to be a useful model for studies of human AIDS chemotherapy due to

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the similarity to human immunodeficiency virus (HIV) infection (Barré-Sinoussi et al., 1983; Gallo et al., 1984). The common feature between the two models includes a high prevalence of specific antibodies in cats with FIV-induced immune deficiency (Pedersen et al., 1987, 1989), a disease entity in cats similar to human AIDS which can be induced experimentally (Egberink et al., 1990; Pedersen et al., 1987, 1989, 1990), a Mg^{2+}-dependent reverse transcriptase (RT) activity (Pedersen et al., 1987), preferential infection of T-lymphocytes and cytolysis of the infected cells (Miyazawa et al., 1989; Pedersen et al., 1987; Tochikura et al., 1990).

In vitro evaluation of anti-retroviral agents often depends on prevention of primary infection as determined by RT activity from virus-infected culture fluids. Based on RT reduction, the most promising anti-retroviral agents are nucleoside analogues such as 3′-azido- 2′3′-dideoxythymidine (AZT) or 2′,3′-dideoxycytidine whose corresponding nucleotides are selectively targeted against the transcriptional operation (Egberink et al., 1990; North et al., 1989; Remington et al., 1991). However, those agents do not interfere with a very early stage of the virus replication cycle, presumably virus adsorption (Baba et al., 1990). By contrast, it has been reported that several sulfated polysaccharides are highly selective inhibitors of HIV replication in vitro because of their putative effect on the virus adsorption process (Baba et al., 1988; Baba et al., 1990; Mitsuya et al., 1988; Nakashima et al., 1990).

We report here that anti-HIV agents including sulfated polysaccharides (dextran sulfate and heparin) and a new antifungal antibiotic, pradimicin A, in addition to their inhibitory effect on FIV replication by cell-free infection in the feline lymphoid cell line 3201, are also capable of blocking giant cell formation in co-culture of FIV-infected 3201 cells (3201/FIV) (FIV is the Petaluma strain; Pedersen et al., 1987; provided by The AIDS Research and Reference Reagent Program, Division of AIDS, The National Institute of Allergy and Infectious Diseases, National Institute of Health, Bethesda, MD) and human T-cell lymphotropic virus type I (HTLV-I) producer MT-2 cells.

Dextran sulfate (DS, molecular weights [MW] of 5000 and 500 000), dextran (MW 162 000) and sodium heparin were purchased from Sigma Chemical Co. (St. Louis, MO). Pradimicin A hydrochloride salt (Oki et al., 1988), a new antifungal antibiotic obtained from the culture filtrate of *Actinomadura hibisca* sp. nov. Strain P157-2 (ATCC 53557) was kindly provided by T. Oki at Bristol-Myers Squibb Research Institute (Tokyo, Japan). 3′-Azido-2′3′- dideoxythymidine (AZT) and 2′3′-dideoxyinosine (ddI) were provided by The National Institute of Allergy and Infectious Diseases AIDS Research and Reference Reagent Program, Bethesda, MD. These substances were dissolved in complete medium.

Tissue culture fluids from 3201/FIV cells were used to prepare virus stocks. The virus titer was $10^{1.0}$–$10^{1.5}$ 50% tissue culture infectious dose (TCID 50/ml) assayed in 3201 cells as calculated by the method of Reed and Muench (1938).

The cell lines were obtained as follows: a feline lymphoid cell line, 3201 (provided by W. Hardy, Jr., Memorial Sloan Kettering Cancer Center, New

York, NY) (Snyder et al., 1978) and MT-2 originally established by I. Miyoshi et al. (1982) (provided by M. Lairmore, Ohio State University, Columbus, OH) were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 IU/ml) and streptomycin (100 μg/ml). The 3201/FIV cell line (Tochikura et al., 1990) was maintained similarly in complete medium with 5% FBS. The cell lines used for the anti-FIV assay were mycoplasma-negative by monthly examination for mycoplasma contamination by means of the Gen-Probe MYCOPLASMA T. C. Rapid Detection System (Gen-Probe, Inc., San Diego, CA) and subcultured routinely every 4 days at 37°C in 5% CO_2.

Initially, antiviral agents were evaluated for their inhibition of FIV-induced cell killing. 3201 cells were infected with FIV at a multiplicity of infection (M.O.I.) of approximately 0.0001 TCID 50. After adsorption at 37°C for 1 h, infected cells were seeded into a tissue culture flask. The cell number was adjusted to 2×10^5/ml with 10 ml of complete medium containing 5% FBS. The anti-viral agent being tested (AZT, ddI, pradimicin A, or heparin) was dissolved in complete medium and added to the culture at the time of seeding. The cells were subcultured every 4 days and the medium was replaced with fresh growth medium containing drugs. The number of viable cells was determined microscopically by trypan blue dye-exclusion method. Anti-FIV activity of the substances was also monitored by indirect immunofluorescence assay (IFA) using methanol-fixed smeared cells with a 1:100 dilution of serum from a pet cat naturally infected with FIV or control serum from an uninfected SPF cat (Tochikura et al., 1990) and by Mg^{2+}-dependent RT activity determined by [^{3}H]dTTP-incorporation using cell-free culture fluid (Tochikura et al., 1990).

Mg^{2+}-dependent RT activity and FIV antigen-positive cells by IFA were evident beginning at 12 days and 16 days post-inoculation (dpi), respectively. Cell death was observed beginning at 16 dpi, with a maximum of 27% dead at 24 dpi. Cytopathic effect (CPE) began to subside at 28 dpi and at 32 dpi CPE disappeared completely. The remaining cells became positive for FIV antigens as reported previously (Tochikura et al., 1990). The protective effect of the anti-HIV agents on FIV-induced CPE was assessed at 4-day intervals for a period of 32 days. We found that concentrations of > 0.27 μg/ml (1 μM) AZT, > 10 μg/ml pradimicin A and > 10 μg/ml heparin completely protected 3201 cells against FIV-cytopathogenicity. At these concentrations virus antigen expression as determined by IFA and RT activity were completely blocked (Table 1). The antiviral activities of AZT, pradimicin A and heparin expressed as the concentration (μg/ml) required to reduce the RT activity by 50% (IC_{50}) were 0.0076, 0.54 and 0.34, respectively, on day 28. In the same study ddI required much higher concentration than AZT to exert anti-FIV effects ($IC_{50} = 0.26$ μg/ml). Significant growth inhibition of 3201 cells was not observed at the concentrations tested.

Next, DS (MW 5000) and DS (MW 500 000) were examined for their inhibitory effect on the FIV antigen expression and on the cytopathogenicity of

TABLE 1

Inhibition of the infectivity and replication of FIV in 3201 cells by various substances

Concentration (Pradimicin A, Heparin; μg/ml, AZT, ddI; μM)	IF-positive cells (%)				% inhibition of RT activity[a]			
	Pradimicin A	Heparin	AZT	ddI	Pradimicin A	Heparin	AZT	ddI
100	0	0	ND[b]	ND	99.4	99.5	ND	ND
10	0	0	0	0.4	99.5	99.4	99.6	94.0
1	51.0	0.5	0	51.0	24.0	94.2	99.5	25.6
0.1	50.5	ND	0.1	50.3	6.0	ND	96.9	26.8
0.01	ND	ND	52.8	51.6	ND	ND	4.0	10.9
0	52.2				0 (275,799 cpm)			

The effects of the drugs were determined 28 days after infection.
[a]Percentage of inhibition was calculated as the:
ratio = (1 − counts in the presence of inhibitor/counts in the absence of inhibitor) × 100
[b]ND = not done.

FIV, in 3201 cells. In this experiment, control cells were FIV antigen-positive at 6 dpi and cell death was observed as early as 9 dpi, with a maximum of 35% dead at 19 dpi (data not shown). Although both molecular weight ranges of DS exhibited anti-FIV activity, they behaved differently in their potency profiles based on concentration. Neither DS compound was cytotoxic for 3201 cells, as measured by trypan blue dye exclusion, at the concentration used. DS (MW 5000) and DS (MW 500 000) continued to block the spread of FIV at concentrations of 0.1 μg/ml and 10 μg/ml, respectively through day 35 (data not shown).

DS (MW 5000) completely inhibited FIV-antigen expression as determined by IFA and virus-induced cell death at a concentration of 0.1 μg/ml. By comparison, DS (MW 500 000) requires 100 times that concentration to have the same inhibitory activity. Antiviral activities of DS (MW 5000) and DS (MW 500 000), expressed as the concentration (μg/ml) required to reduce the number of FIV antigen-positive cells by 50% (IC_{50}), were 0.01 and 1.0, respectively, on day 16. It should be noted that on a weight per weight basis, the antiviral potency of the two DS preparations, were significantly different, but on a mole per mole basis their potencies were equal.

Additional studies were performed to evaluate the effect of antiviral agents on cell fusion between 3201/FIV and MT-2 cells. In the first series of co-culture experiments we determined the optimal cell ratio of 3201/FIV and MT-2 cells for the development of cell fusion. The two types of cells were mixed at different ratios (final cell density = 5×10^5 cells/ml), and the appearance of giant cells was followed kinetically in the presence of complete medium with 10% FBS. The results showed that, among the cultures with various cell ratios, when 3201/FIV and MT-2 cell were mixed in a ratio of 1:1, multinucleated giant cells were most clearly observed and became apparent within 2 h after co-cultivation (Fig. 1D and 2). Staining of multinucleated giant cells for human

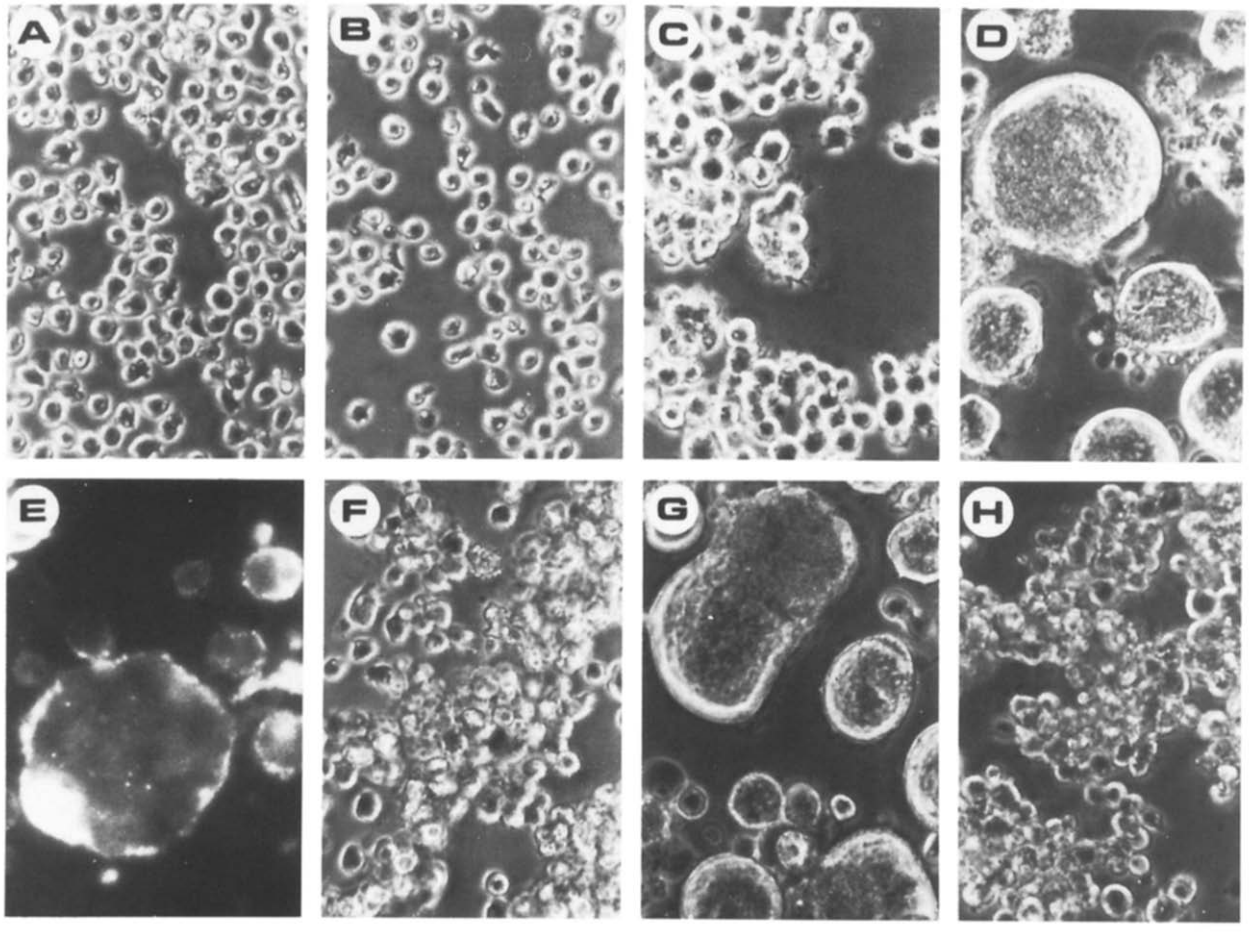

Fig. 1. Morphological changes after cocultivation (D-H) of MT-2 cells and 3201/FIV cells (D, E, G and H) or 3201 cells (F). MT-2 and 3201/FIV or 3201 cells were mixed 1:1 in proportion to cell number and were seeded to individual wells by adjusting to a final concentration of 5×10^5 cells/ml in the presence of AZT (G) and pradimicin A (H) or in the absence of drug (D). After 10 h incubation, cells were photographed by light microscopy (A–D and F–H, original magnification, $\times 200$) or by immunofluorescent microscopy (E, $\times 200$) after being stained with phycoerythrin (PE)-conjugated anti-CD4 mouse monoclonal antibody Leu 3a. For IFA, washed cells were incubated with 20 μl of PE-conjugated Leu 3a (Becton Dickinson). Cells were washed once, resuspended in a 1:1 mixture of PBS:glycerol and examined for membrane immunofluorescence.

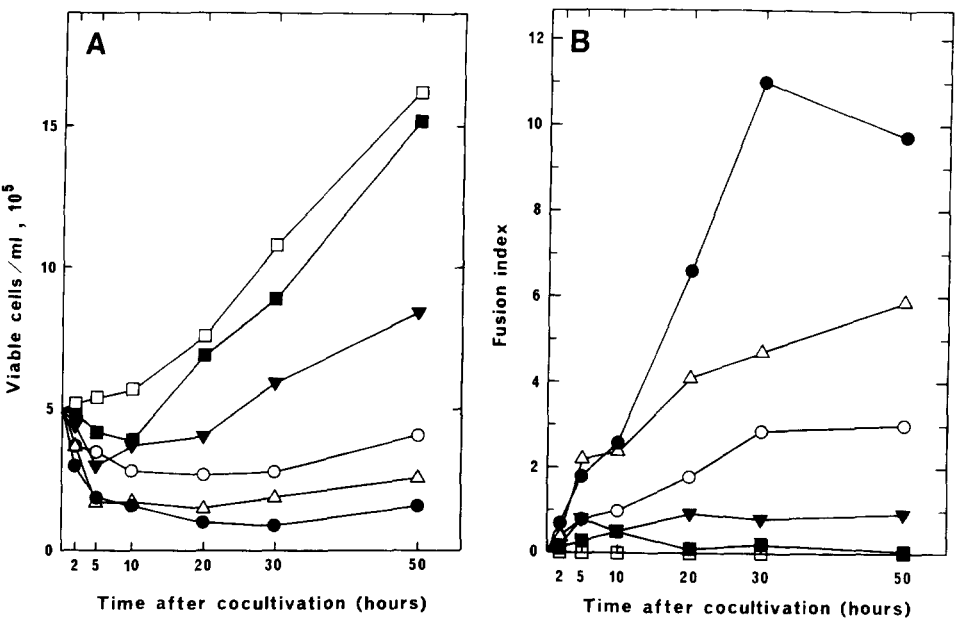

Fig. 2. Effect of different cell ratios on cell number (A) and fusion index (B) in cocultivation of MT-2 and 3201/FIV cells. Cells were mixed 10:0 (□), 9:1 (■), 7:3 (○), 1:1 (●), 3:7 (△) and 1:9 (▼) in proportion to cell number by adjusting to 5×10^5 cells/ml. Cell number was determined by a trypan blue dye exclusion method.

CD4 antigens using anti-CD4 monoclonal antibody demonstrated bright fluorescence on the cytoplasmic membrane surfaces (Fig. 1E). Similarly, these giant cells, but not MT-2 cells alone, stained with anti-FIV antibody (data not shown). 3201/FIV cells alone stained FIV-positive but failed to stain with the anti-human CD-4 antibody. The results suggest that the multinucleated giant cells were a fusion of human and feline cells. No giant cells were observed when MT-2 cells were cocultivated with uninfected 3201 cells (Fig. 1A). The decrease of the total cell number was due to a shift of ordinary-sized cells to giant cells, which serves as an indicator of cell fusion (Fig. 2A). Using this indicator, we attempted to quantify cell fusion induced by FIV. For this purpose we applied the fusion index (FI), which has been used in quantitative analysis of the fusion phenomenon by HIV (Tochikura et al., 1988).

The calculation was done as follows:

$$\mathrm{FI} = \frac{\text{Cell number in MT}-2\text{ control well}}{\text{Cell number in coculture (MT}-2+3201/\mathrm{FIV})\text{ well}} - 1.0$$

The control well was cultured only with MT-2 cells. The FI of the control expressed in this form is zero. As cell fusion progresses over the 30-h

observation period the numerator of FI decreases causing an increase in the value of FI.

As shown in Fig. 2B, at a cell ratio of 1:1 the FI increased remarkably within 2 h and reached a maximum 30 h after cocultivation. The increase in FI value was consistent with morphological appearance in microscopic observations. That is to say, a cell ratio of 1:1 was most efficient in forming cell fusion as compared to cultures with other cell ratios. Thus, this cell ratio was chosen for further experiments.

FIV-infected cats have antibodies against the viral proteins of FIV. Therefore, we next examined whether giant cell formation could be inhibited by these antibodies. Serum samples including antisera against FIV derived from naturally infected cats and human sera with antibody against HIV (IFA titer = 1:640), or against HTLV-1 (IFA titer = 1:640) were heated for 45 min at 56°C before use. Sera from FIV-infected cats inhibited giant cell formation completely at 1:100 dilution. By contrast, no inhibitory activity was detected with serum samples prepared from HIV or HTLV-1 patients, or normal SPF cats, or with OKT4a monoclonal antibody (10 μg/ml, Ortho Diagnostic Systems) which recognizes the HIV-binding site on the CD 4 molecule (McDougal et al., 1986).

To show that the inhibitor factor was immunoglobulin, sera from FIV-infected cats (IFA titer, > 2560) which inhibited cell fusion were adsorbed with Staph protein A. This was done by resuspension of pelletized killed *Staphylococcus aureus* in the serum sample, incubation for 1 h at 37°C and centrifugation at 1200 × *g* for 30 min. Protein A adsorption of the sera removed all fusion inhibition activity.

To determine the source of the fusion-inducing ligand, FIV-infected cat sera were adsorbed with 3201, MT-2 or 3201/FIV cells. Cells used for adsorption were resuspended in 1 ml culture medium (2×10^8 cells/ml) containing 1:100 dilution of antisera. After 1 h incubation at 37°C the cells were pelleted at 3000 × *g* for 30 min and the supernatant filtered through a 0.45-μm filter. Only the 3201/FIV cells removed the fusion inhibition activity of antiserum, suggesting that the antibodies were specific for FIV antigen.

Incidently, RD114 virus, first isolated as a typical type C oncornavirus from a human cell line and subsequently identified as an endogenous virus of the domestic cat, has been shown to induce syncytia in a human malignant glioma cell line transformed by Rous sarcoma virus (Rand and Long, 1973). To rule out the possibility that our FIV stocks contained RD-114 and that RD-114 was mediating cell fusion instead of FIV, antiserum to RD-114 virus was used in the giant cell inhibition test. In this experiment, goat anti-RD 114 virus, at non-toxic concentration, did not block the giant cell formation. In addition, purified cell-free concentrated RD114 virus (1.1×10^{12} virus particles/ml by electron microscopy) from Pfizer, Inc. (Maywood, NJ) did not induce MT-2 cells to form syncytium (data not shown).

We then studied various substances which are known to block HIV-induced giant cell formation. MT-2 and 3201/FIV cells were mixed in a ratio of 1:1 and

TABLE 2

Effect of various drug on giant cell formation after cocultivation of MT-2 and 3201/FIV cells

Concentration of drug (μg/ml)		Time after cocultivation[a] (h)					
		2		10		30	
		FI[b]	Viable cells ($\times 10^5$/ml) Viability (%)	FI	Viable cells ($\times 10^5$/ml) Viability (%)	FI	Viable cells ($\times 10^5$/ml) Viability (%)
None		1.30 (0)[c]	2.3 (82.1)	2.53 (0)	1.7 (73.9)	11.00 (0)	0.9 (52.9)
AZT	2.7	0.60 (53.8)	3.3 (94.3)	1.86 (26.5)	2.1 (67.7)	9.80 (10.9)	1.0 (55.6)
ddI	2.4	0.33 (74.6)	4.0 (93.0)	2.33 (7.9)	1.8 (78.3)	9.80 (10.9)	1.0 (55.6)
Pradimicin A	10	0 (100)	5.6 (91.8)	0.02 (99.2)	5.9 (90.8)	0.38 (96.5)	7.8 (91.8)
	5	0.04 (96.9)	5.1 (92.7)	0.15 (94.1)	5.2 (88.1)	0.61 (94.5)	6.7 (90.5)
	1	0.77 (40.8)	3.0 (83.3)	2.16 (14.6)	1.9 (79.2)	8.00 (27.3)	1.2 (44.4)
Heparin	100	0.36 (72.3)	3.9 (86.7)	4.00 (0)	1.2 (70.6)	8.82 (19.8)	1.0 (47.8)
Dextran sulfate (MW:5000)	1000	0.10 (92.3)	4.8 (94.1)	0.13 (94.9)	5.3 (91.4)	0.29 (97.4)	8.4 (92.3)
	100	0.33 (74.6)	4.0 (93.0)	0.76 (70.0)	3.4 (87.2)	1.20 (89.1)	4.9 (89.1)
	10	0.39 (70.0)	3.8 (95.0)	1.22 (51.8)	2.7 (87.1)	4.68 (57.5)	1.9 (73.1)
	1	0.43 (66.9)	3.7 (84.1)	1.86 (26.5)	2.1 (75.0)	8.00 (27.3)	1.2 (57.1)
Dextran sulfate (MW:500 000	100	0 (100)	5.5 (91.7)	0.09 (96.5)	5.5 (91.7)	0.17 (98.5)	9.2 (90.2)
	10	0.06 (95.4)	5.0 (92.6)	0.15 (94.1)	5.2 (91.2)	0.42 (96.2)	7.6 (90.5)
	1	0.02 (98.5)	5.2 (86.7)	1.73 (31.6)	2.2 (71.0)	8.82 (19.8)	1.1 (52.4)

[a]MT-2 and 3201/FIV cells were mixed 1:1 in proportion to cell number by adjusting to final concentration of 5×10^5 cells/ml.
[b]The fusion index (FI) of control culture (ratio of MT-2 and 3201/FIV cells in 10:0) is zero.
[c]Percent of inhibition.

the mixed cell suspension was cultured with various concentrations of the antiviral agents and FI was calculated from samples collected 2, 10 and 30 h after co-culture. If pradimicin A ($>$ 5 μg/ml), DS (MW 5000:1 mg/ml), DS (MW 500 000: $>$ 10 μg/ml) were added to the co-culture of 3201/FIV and MT-2 cells, giant cell formation was inhibited by $>$90% (Fig. 1H and Table 2). Antiviral activities of these compounds expressed as the concentration (μg/ml) required to reduce the value of FI by 50% (IC_{50}) were calculated to be 1.0, 7.8 and 2.0, respectively (Table 2, 30 h). Furthermore, the value of the FI increased with decreasing concentrations of pradimicin A, indicating dose-dependent inhibition of cell fusion by the agent. For pradimicin A (5 μg/ml), the FI of the culture was more than 15-fold lower than that with the two nucleoside analogues, AZT and ddI, and control where no agents were added (Table 2). DS also proved inhibitory to giant cell formation, albeit less so than pradimicin A. However, for DS (MW 5000), a concentration of 1000 μg/ml which is 10 to

100-fold higher than that of DS (MW 500 000) was required to inhibit >90% of FI, indicating that the anti-FIV activity of DS (MW 500 000) is superior to that of DS (MW 5000), when determined by cell fusion inhibition. With heparin, no significant inhibition of giant cell formation was observed, even at a concentration of 1 mg/ml (data not shown).

So far, only nucleoside analogues have unambiguously demonstrated both in vitro benefit in the treatment of FIV-infected cells (Egberink et al., 1990; North et al., 1989; Remington et al., 1991) and clinical benefit (Egberink et al., 1990). Anti-HIV agents used in this report whose mechanism seems to be interference with an early event of the virus replicative cycle, presumably virus adsorption, were also shown to be potent and selective inhibitors of FIV replication in vitro by cell-free infection with cat T-cell line, 3201. Next, we demonstrated that co-cultivation of the FIV-producing cell line, 3201/FIV with HTLV-I-positive human T-cell line, MT-2 resulted in the formation of large, multinucleated giant cells. Although the experiments presented here did not demonstrate which component of FIV-infected cells induced giant cell formation, we suspect that the cell fusion was induced by a virion envelope protein such as the glycoprotein, because adsorption with 3201/FIV cells removed the blocking activity of giant cell formation, while adsorption with 3201 cells or MT-2 cells did not. The giant cell formation assay described is very similar to that developed by Tochikura et al. for the quantitation of HIV (Tochikura et al., 1988). In our cell-free infection experiment, the concentration of AZT (0.027 to 0.27 μg/ml) required to inhibit replication of FIV is similar to that required to inhibit replication of FIV (Remington et al., 1991) and HIV (Mitsuya et al., 1985; Nakashima et al., 1990), previously reported. Moreover, as reported in FIV and HIV studies, ddI was less effective than AZT (Nakashima et al., 1990). However, AZT and ddI failed to block the giant cell formation by FIV. This is expected since these nucleoside analogues do not affect the interaction of the HIV envelope protein with its receptor CD4. Unlike AZT, ddI and other dideoxynucleoside analogues (Baba et al., 1990; Tochikura et al., 1988), sulfated polysaccharides proved capable of blocking giant cell formation not only by HIV but also by FIV. This observation points to a potentially important therapeutic advantage of the sulfated polysaccharides over dideoxynucleoside analogues. Because of their marked in vitro activity against HIV (Baba et al., 1988; Baba et al., 1990; Mitsuya et al., 1988), DS has been introduced in the clinic for the treatment of AIDS (Abrams et al., 1989). However, not all sulfated polysaccharides were inhibitory to giant cell formation, i.e. heparin was markedly active against FIV replication but did not inhibit giant cell formation even at a concentration of 1 mg/ml. This result is in agreement with HIV study (Baba et al., 1990). In the present study, the inhibitory concentration (μg/ml) of DS (MW 500 000) for giant cell formation ($IC_{50} = 2.0$) was almost the same as that for FIV replication ($IC_{50} = 1.0$). By comparison, DS (MW 5000) was 100 times less effective at inhibiting giant cell formation but 100 times more effective at preventing virus replication than DS (MW 500 000). On a mole per mole basis, DS (MW 5000) was 1000 to 10 000

times less effective than DS (MW 500 000) at preventing giant cell formation but equal in preventing virus replication. These results may be due to the size difference between the two DS species, the small molecular weight form, DS (MW 5000), being equal or more effective at preventing virus infection and replication. The degree of sulfation may also be important in blocking cell fusion, as reported in HIV study (Nakashima et al., 1987). We have demonstrated that dextran (MW 162 000) has no antiviral activity in the FIV system (data not shown). A novel antifungal antibiotic, pradimicin A has been shown to inhibit an early step in HIV infection, probably through its binding to mannose residues of HIV glycoprotein (Tanabe-Tochikura et al., 1990). It has also been reported that pradimicin A exhibited a marked in vivo therapeutic activity against systemic fungal infections caused by *Candida albicans, Aspergillus fumigatus,* and *Cryptococcus neoformans* strains in mice (Oki et al., 1990). Fungi, such as *Candida, Cryptococcus,* or *Aspergillus* have been frequently detected in patients with AIDS and FIV-infected cats in the immune deficiency phase of illness (Pedersen et al., 1989). In view of the high selectivity with low dosage of pradimicin A as inhibitors of HIV and FIV replication in vitro, this kind of agent targeted to sites other than RT may be the promising candidate used individually or in combination for further testing in vivo, using animal models for AIDS chemotherapy.

This quantitative procedure is indeed simple and rapid. Several substances which were known to inhibit HIV-induced cell fusion and cell-free HIV infection, were effective against FIV infection by our procedure, suggesting that this co-cultivation method may be useful for the screening of anti-FIV agents.

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