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The mannose-specific plant lectins from *Cymbidium* hybrid and *Epipactis helleborine* and the (*N*-acetylglucosamine)$_n$-specific plant lectin from *Urtica dioica* are potent and selective inhibitors of human immunodeficiency virus and cytomegalovirus replication in vitro

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Summary

A series of four mannose(Man)-, three *N*-acetylglucosamine (GlcNAc)$_n$-, ten N-acetylgalactosamine/galactose(GalNAc/Gal)-, one 5-acetylneuraminic acid(α-2,3-Gal/GalNAc)- and one 5-acetylneuraminic acid(α-2,6-Gal/GalNAc)-specific plant agglutinins were evaluated for their antiviral activity in vitro. The mannose-specific lectins from the orchid species *Cymbidium* hybrid (CA), *Epipactis helleborine* (EHA) and *Listera ovata* (LOA) were highly inhibitory to human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) in MT-4, and showed a marked anti-human cytomegalovirus (CMV), respiratory syncytial virus (RSV) and influenza A virus activity in HEL, HeLa and MDCK cells, respectively. The 50% effective concentration (EC_{50}) of CA and EHA for HIV ranged from 0.04 to 0.08 μg/ml, that is about 3 orders of magnitude below their toxicity threshold (50% inhibitory concentration for MT-4 cell growth: 54 to 60 μg/ml). Also, the (GlcNAc)$_n$-specific lectin from *Urtica dioica* (UDA) was inhibitory to HIV-1-, HIV-2-, CMV-, RSV- and influenza A virus-induced cytopathicity at an EC_{50} ranging from 0.3 to 9 μg/ml.

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The GalNAc/Gal-, α-2,3-Gal/GalNAc- or α-2,6-Gal/GalNAc-specific lectins were not inhibitory to HIV or CMV at non-toxic concentrations. CA, EHA and UDA proved to be potent inhibitors of syncytium formation between persistently HIV-1- and HIV-2-infected HUT-78 cells and $CD4^+$ Molt/4 (clone 8) cells (EC_{50}: 0.2–2 μg/ml). Unlike dextran sulfate, the plant lectins CA, EHA and UDA did not interfere with HIV-1 adsorption to MT-4 cells and RSV- and influenza A virus adsorption to HeLa and MDCK cells, respectively. They presumably interact at the level of virion fusion with the target cell.

Plant lectins; AIDS; Human immunodeficiency virus (HIV); Cytomegalovirus (CMV); Viral fusion

Introduction

Lectins are proteins that are able to bind to the oligosaccharide (glycan) side chains linked to a peptide backbone of the glycoproteins via asparagine or serine/threonine. The binding between lectins and glycans depends on a highly specific molecular interaction. Since the envelope glycoprotein of HIV is heavily glycosylated, one may expect human immunodeficiency virus (HIV) infectivity and virus–cell fusion to be inhibited by those lectins that are specific for the glycans present in the gp120 molecule. Lifson et al. (1986) and Hansen et al. (1989) showed that lectins with specificity for different glycan structures are able to block fusion of HIV-infected cells with $CD4^+$ cells. In particular, D-mannose-specific lectins such as concanavalin A (ConA) (Lifson et al., 1986) and *Gerardia savaglia* agglutinin (GSA) (Muller et al., 1988) block virion infectivity and cell fusion at concentrations as low as 0.2 μM. Using a panel of 13 different lectins, Hansen and co-workers (1989) investigated the glycans present in gp120, and found at least 4 different types of gp120 glycans that can be recognized by lectins. Lectins directed against one of these 4 glycan types are able to inhibit CD4-gp120 association and subsequent syncytium formation.

We have now evaluated a panel of 4 Man-, 3 GlcNAc- and 12 GalNAc-specific plant lectins for their activity against several retroviruses, herpes viruses, respiratory syncytial virus and influenza A virus and found that the mannose-specific lectins from the orchids *Listera ovata*, *Cymbidium* hybrid and *Epipactis helleborine*, and the $(N$-acetylglucosamine$)_n$-specific lectin from *Urtica dioica* are particularly potent and selective inhibitors of HIV-induced cytopathicity in MT-4 cells. These plant lectins are assumed to confer their antiviral effect by interference with the fusion between the virion and the target cell membrane.

Materials and Methods

Compounds

The origin, purification and specificity of the lectins used in our study are summarized in Table 1. All lectins were purified by affinity chromatography on carbohydrates or fetuin immobilized to Sepharose 4B followed in some cases by hydrophobic interactions chromatography on phenyl Sepharose and/or ion exchange chromatography on either Q Fast Flow or S Fast Flow (Pharmacia-LKB, Uppsala, Sweden). The purification of the lectins from *Cymbidium* hybrid and *Epipactis helleborine* has not been described yet. However, the procedure followed was essentially the same as that described for the isolation of the *Listera ovata* agglutinin (Van Damme et al., 1987). Dextran sulfate (molecular weight 5000 and 10000) (DS-5000, DS-10000) was obtained from Sigma Chemical Company (St. Louis, MO), and 9-(1,3-dihydroxy-2-propoxy-methyl)guanine (DHPG) was from Syntex (Palo Alto, CA).

Viruses

HIV-1 (strain HTLV-III_B) was originally obtained from the culture supernatant of the persistently HIV-infected H9 cell line (H9/HTLV-III_B) (Popovic et al., 1984) and was kindly provided by Dr. R.C. Gallo (National Cancer Institute, National Institutes of Health, Bethesda, MD). HIV-2 (strain LAV-2) (Clavel et al., 1986) was a gift from Dr. L. Montagnier (Pasteur Institute, Paris, France). Simian immunodeficiency virus (SIV) (strain SIV_{Mac251}) was originally isolated by Daniel et al. (1985) and obtained from Dr. C. Bruck (Smith Kline-Rit, Rixensart, Belgium). Moloney murine sarcoma virus (MSV) was prepared from tumors induced following intramuscular inoculation of 3-day-old NMRI mice with MSV, as described previously (De Clercq and Merigan, 1971). Human cytomegalovirus (CMV) (strains AD169 and Davis) was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and kindly provided by Dr. S. Michelson (Pasteur Institute, Paris, France). The preparation of respiratory syncytial virus (RSV) (Long strain) and influenza virus type A [A/Ishikawa/7/82(H3N2)] has been previously described (Kawana et al., 1987; Shigeta et al., 1988).

Anti-retrovirus assays

The methodology of the anti-HIV and -SIV assays has been described previously (Balzarini et al., 1989, 1990). Briefly, MT-4 cells (4.5×10^5 cells/ml) were suspended in fresh culture medium and infected with HIV-1, HIV-2 or SIV at 100 $CCID_{50}$ per ml cell suspension (1 $CCID_{50}$ being the dose infective for 50% of the cell cultures). Then, 100 μl of the infected cell suspension was transferred to microplate wells, mixed with 100 μl of the appropriate dilutions of the test compounds, and further incubated at 37°C. After 5 days, the number of viable cells was determined in a blood cell-counting chamber by Trypan blue staining.

The effects of the test compounds on MSV-induced transformation of

TABLE 1

Abbreviations, sources of isolation and purification method, molecular weights and references of the plant lectins

GlcNAc-specific agglutinins
WGA: Wheat germ agglutinin/wheat germs/immobilized GlcNAc; M.W.: 2 × 18 kDa) (Peumans et al., 1982)

$(GlcNAc)_n$-specific agglutinins
STA: *Solanum tuberosum* agglutinin/potato tubers/immobilized $(GlcNAc)_n$; M.W.: 2 × 50 kDa (Desai and Allen, 1979)
UDA: *Urtica dioica* agglutinin/stinging nettle rhizomes/immobilized $(GlcNAc)_n$; M.W.: 8 kDa (Peumans et al., 1984)

GalNAc/Gal-specific agglutinins
APA: *Aegopodium podagraria* agglutinin/ground elder rhizomes/immobilized human erythrocyte membrane glycoproteins; M.W.: 8 × 60 kDa (Peumans et al., 1985)
BDA: *Bryonia dioica* agglutinin/white bryony root stocks/immobilized fetuin; M.W.: 2 × 30 kDa (Peumans et al., 1984)
BPA: *Bauhinia purpurea* agglutinin/camel's foot tree seeds/immobilized GalNAc; M.W.: 4 × 32 kDa (Osawa et al., 1978)
DBA: *Dolichos biflorus* agglutinin/horse gram seeds/immobilized GalNAc; M.W.: 4 × 26 kDa (Etzler, 1973)
EHL: *Eranthis hyemalis* agglutinin/winter aconite tubers/immobilized fetuin; M.W.: 30 + 32 kDa (Cammue et al., 1985)
IRA: *Iris reticulata* agglutinin/iris bulbs/immobilized fetuin; M.W.: not determined (unpublished)
RPA: *Robinia pseudoacacia* agglutinin/black locust bark/immobilized fetuin; M.W.: (2 × 29 kDa) + (2 × 31 kDa) (Peumans et al., 1986)
RSA: *Rhizoctonia solani* agglutinin/rhizoctonia solani sclerotes/immobilized galactose; M.W.: 2 × 13 kDa (Vrancken et al., 1987)
SBA: Soybean agglutinin/soybean seeds/immobilized GalNAc; M.W.: 4 × 30 kDa (Allen and Neuberger, 1975)
SNAII: *Sambucus nigra* agglutinin II/elderberry bark/immobilized GalNac; M.W.: 2 × 30 kDa (Kaku et al., 1990; Shibuya et al., 1987)

Mannose-specific agglutinins
AUA: *Allium ursinum* agglutinin/ramsons bulbs/immobilized mannose; M.W.: 12 + 13 kDa (Van Damme et al., 1991)
CA: *Cymbidium* hybrid agglutinin/cymbidium leaves/immobilized mannose; M.W.: not determined (unpublished)
EHA: *Epipactis helleborine* agglutinin/broad-leaved helleborine leaves/immobilized mannose; M.W.: not determined (unpublished)
LOA: *Listera ovata* agglutinin/twayblade leaves/immobilized mannose; M.W.: 2 × 12.5 kDa (Van Damme et al., 1987)

Neu5Ac(α-2,3-Gal/GalNAc)-specific agglutinins
MAA: *Maackia amurensis* agglutinin/maackia bark/immobilized fetuin; M.W.: (2 × 33 kDa) + (2 × 37 kDa) (Wang and Cummings, 1988)

Neu5Ac(α-2,6-Gal/GalNac)-specific agglutinins
SNA I: *Sambucus nigra* agglutinin I/elderberry bark/immobilized fetuin; M.W.: (2 × 34.5 kDa) + (2 × 37.5 kDa) (Broekaert et al., 1984)

murine C3H/3T3 cells were examined on day 6 post infection, as described previously (Balzarini et al., 1987).

Anti-viral assays

Activity against herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), vaccinia virus (VV) and vesicular stomatitis virus (VSV) was determined by microscopic examination of virus-induced cytopathicity in E_6SM cell cultures, as previously described (De Clercq, 1985; De Clercq et al., 1989).

Anti-cytomegalovirus assays

Confluent human embryonic lung (HEL) cells were infected with human cytomegalovirus (CMV) (strain AD169 or strain Davis). The virus was diluted in cell culture medium containing 2% fetal calf serum (FCS) and different concentrations of the plant lectins. After a 2-h incubation period at 37°C, the virus was removed and fresh medium containing the different drug concentrations was added to the cells. After 7 days incubation, virus-induced cytopathic effect was monitored microscopically following ethanol fixation and Giemsa staining.

Co-cultivation assays

Persistently HIV-1- or HIV-2-infected HUT-78 cells (designated HUT-78/HIV-1 and HUT-78/HIV-2, respectively) were washed to remove free virus from the culture medium, and 5×10^4 cells (50 μl) were transferred to 96-well microtiter plates (Sterilin). Then, 5×10^4 Molt-4 (clone 8) cells (50 μl) together with an appropriate concentration of the test compounds (100 μl) were added to each well. The mixed cells were cultured at 37°C in a CO_2-controlled atmosphere. The first syncytia were noted after about 3–4 h co-cultivation. After 16–20 h, syncytium formation reached its maximum, and the number of syncytia were counted under the microscope.

Interference of plant lectins with binding of HIV-1 particles to CD4+ cells

HIV-1 was incubated with appropriate concentrations of UDA and EHA or DS-5000 for 2 h prior to addition of the virus suspension to MT-4 cells. The cell cultures were then incubated for 30 min at 37°C and washed twice with PBS to remove unbound virus. Then, a high-titer polyclonal antibody derived from a patient with AIDS-related complex was added, and after another 30 min incubation at room temperature, the cells were washed twice with PBS, treated with FITC-conjugated F(ab')2 fragments of rabbit anti-human immunoglobulin antibody [RaH-IG-F(ab')2-FITC] (Dako) for 30 min at room temperature and assayed by FACS analysis with a fluorescence-activated cell sorter (FACSTAR, Becton-Dickinson).

Interference of plant lectins with binding of anti-gp120 mAb to HUT-78/HIV-1 cells

HUT-78/HIV-1 cells were washed and incubated with DS-5000 (25 μg/ml) or plant lectins (40, 20, 4 or 0.8 μg/ml) for 15 min. The cell cultures were then incubated with anti-gp120 mAb (NEA 9284) (E.I. du Pont de Nemours), washed twice with cell culture medium, incubated with rabbit anti-mouse

immunoglobulin G (IgG)-F(ab')$_2$-fluorescein isothiocyanate (Dako), washed twice in phosphate-buffered saline (PBS), resuspended in 0.37% paraformaldehyde in PBS and analyzed by FACS analysis. The threshold of positivity for the green fluorescence intensity was arbitrarily established on the basis of the control sample of uninfected HUT-78 cells incubated with mAb and RAM-IgG-F(ab')$_2$-FITC. The percentage inhibition of mAb binding was calculated according to the formula: 100 × [1-(Cx-Co)/(C-Co)], whereby Co is the percentage of fluorescent cells in uninfected HUT-78 cell cultures, C is the percentage of fluorescent cells in persistently HIV-1-infected HUT-78 cells, and Cx is the percentage of fluorescent cells in persistently HIV-1-infected HUT-78 cells exposed to the test compound at concentration x.

Incubation of MT-4 cells with plant lectins at 4°C or 37°C during the HIV-1 adsorption period

In a first set of experiments, MT-4 cells were seeded in 200-μl wells of a 96-well microtiter plate in the presence or absence of appropriate concentrations of the lectins LOA, EHA and UDA, or dextran sulfate (MW 5000). To the cell cultures were then added a concentrated amount of HIV-1 and the infected cells were incubated at either 4°C or 37°C for 120 min. The cell cultures that had been kept at 4°C during the initial infection event were then brought at 37°C, and, together with the other set of HIV-1-infected cell cultures, further incubated at 37°C for 2 days in the presence of the test compounds. Then, HIV-1-specific antigen expression was determined by cytofluorometry as previously described (Balzarini et al., 1991). In a second set of experiments, MT-4 cell cultures were infected with a concentrated amount of HIV-1 in the presence of appropriate concentrations of the test compounds either at 4°C or 37°C for 90 min, but then thoroughly washed with culture medium to carefully remove the test compounds before further incubation at 37°C. At day 5 of the experiments, viable cell number was determined microscopically by Trypan blue dye exclusion.

Effect of the incubation time and temperature on the anti-respiratory syncytial virus (RSV) and influenza A virus activity of DS-5000, LOA and UDA

The procedure for measuring anti-myxovirus activity has been described previously (Hosoya et al., 1989, 1991). The culture medium of confluent HeLa (for RSV) or Madin-Darby canine kidney (MDCK) (for influenza A) cell cultures in 24-well plates was removed and changed for 0.2 ml of maintenance medium with or without the test compounds and 0.2 ml of virus suspension containing 50 PFU. After 1 h incubation at 4°C, medium containing unadsorbed virus was removed and the cell cultures were washed twice with Eagle's MEM. Then, 1 ml of maintenance medium with or without the test compound, containing 0.6% agarose, was added to the cell cultures. After 5 days of incubation at 35°C, the number of virus plaques was determined and the antiviral activities of the test compounds expressed as their 50% effective concentrations (EC_{50}).

Results

Antiviral activity of plant lectins

Four Man-, three $(GlcNAc)_n$-, ten GalNAc/Gal-, one Neu5Ac(α-2,3- Gal/GalNAc)- and one Neu5Ac(α-2,6-Gal/GalNAc)-specific plant lectins were evaluated for their inhibitory effect against retrovirus (i.e. HIV-1, HIV-2, SIV, MSV)-, DNA virus (HSV-1, HSV-2, VV and CMV)- and RNA virus (VSV)-induced cytopathicity in vitro (Tables 2, 3 and 4). The GalNAc/Gal-, Neu5Ac(α-2,3-Gal/GalNAc)- and Neu5Ac(α-2,6-Gal/GalNAc)-specific plant lectins did not show any anti-retrovirus or anti-CMV activity in vitro at subtoxic concentrations (Tables 2 and 3). However, several plant lectins proved inhibitory to HSV-1 and HSV-2 replication in E_6SM cells at concentrations below their toxicity threshold. In particular, BPA and EHL proved to be selective antiherpetic agents, as demonstrated by a selectivity index (ratio MCC/EC_{50}) of 20 to 40 (Table 4). In contrast, the $(GlcNAc)_n$-specific agglutinin (lectin) from *Urtica dioica* (UDA) ($n = 1$) inhibited both HIV-1, HIV-2 and SIV-induced cytopathicity in MT-4 cells at 0.9 to 4.2 μg/ml and HSV-1- and HSV-2-induced cytopathicity at 20 and 7 μg/ml, respectively (Tables 2 and 4). The other $(GlcNAc)_n$-specific lectins (i.e. STA and WGA) showed only marginal, if any, antiviral effect at subtoxic concentrations. However, all three $(GlcNAc)_n$-specific lectins were endowed with a marked anti-CMV activity at a concentration that was well below their toxicity threshold (Table 3). The selectivity index (ratio CC_{50}/EC_{50}) of UDA, STA and WGA against CMV was 114, 74 and 5.5, respectively. In this respect, UDA proved more potent, and equally selective against CMV as DHPG, the current drug of choice in the treatment of CMV infections.

All four mannose-specific plant lectins investigated (i.e. CA, EHA, LOA and AUA) proved highly effective in inhibiting HIV-1-, HIV-2- and SIV-induced cytopathicity in MT-4 cells. Their EC_{50} values ranged from 0.04 to 2.7 μg/ml. As a rule, the Man-specific plant lectins were equally inhibitory to HIV-1 and HIV-2, but 5- to 10-fold less inhibitory to SIV. MSV-induced transformation of murine C3H/3T3 cells was not effected by CA, EHA and AUA at subtoxic concentrations, while LOA inhibited MSV replication at an EC_{50} of 8.9, that is at a 10-fold lower concentration than its EC_{50} against C3H/3T3 cells in vitro (Table 2). While LOA was equally inhibitory to CMV replication in HEL cells as DHPG (1.1 and 0.8 μg/ml, respectively), CA and EHA were at least 10-fold less effective against CMV in vitro. Consequently, the selectivity index of CA and EHA was 5-fold lower than that of LOA and DHPG (Table 3).

Inhibition of syncytium formation between HUT-78/HIV-1 or HUT-78/HIV-2 cells and Molt/4 (clone 8) cells by plant agglutinins

The plant agglutinins were evaluated for their inhibitory effect on syncytium formation between persistently HIV-1- and HIV-2-infected HUT-78 cells (designated HUT-78/HIV-1 or HUT-78/HIV-2) and uninfected $CD4^+$-Molt/4 (clone 8) cells (Table 2). As a rule, none of the GalNAc-, Neu5Ac(α-2,3-Gal/

TABLE 2

Anti-retrovirus activity of plant lectins

Compound	EC_{50}[a] (μg/ml)						CC_{50}[b] (μg/ml)	MCC[c] (μg/ml)
	HIV-1	HIV-2	SIV	MSV	HUT-78/ HIV-1 + Molt-4	HUT-78/ HIV-2 + Molt-4		
Man-specific plant lectins								
CA	0.08	0.06	0.5	>20	0.8	2.0	54	20
EHA	0.04	0.04	0.4	>4	0.4	0.8	60	20
LOA	0.3	0.1	0.7	8.9	0.7	1.0[e]	52	100
AUA	0.85	0.84	2.7	>20	7	–	>100	100
GlcNAc-specific plant lectins								
WGA	>0.8	>4	>4	>4	>20	>20	1.5	20
$(GlcNAn)_n$-specific plant lectins								
STA	>4	>4	>4	>100	>100	>100	5.8	>100
UDA	0.9	1.9	4.2	⩾20	1	2	24	100
GalNac/Gal-specific plant lectins								
APA	>100	>100	>100	>100	>100	>100	>100	>100
BDA	>40	>40	>40	>20	>100	>100	61	100
BPA	>0.8	>0.8	>0.8	>0.16	>100	>100	1.2	0.8
DBA	>20	>20	>20	>4	>100	>100	21	20
EHL	>0.2	>0.2	>0.2	>0.03	>20	>20	0.11	0.16
IRA	>0.16	>0.16	>0.16	>0.8	>100	>100	0.15	0.8
RPA	>4	>4	>4	⩾20	>4	>4	3.1	100
RSA	>4	>4	>4	>4	>100	>100	3.8	4
SBA	>100	>100	>100	>100	>100	>100	>100	>100
SNA II	>4	>4	>4	>20	>100	>100	2.6	100
Neu5Ac(α-2,3-Gal/GalNAc)-specific plant lectins								
MAA	>0.8	>0.8	>0.8	>4	>100	>100	1.2	20
Neu5Ac(α-2,6-Gal/GalNAc)-specific plant lectins								
SNA I	>4	>4	>4	>100	>100	>100	4.6	>100
DS-10 000	0.4	–	1.2	14[d]	25	25	>100	>100[d]

[a] Concentration required to inhibit HIV-1, HIV-2 or SIV-induced cytopathicity in MT-4 cells, or MSV-induced transformation of murine C3H/3T3 cells, or giant cell (syncytium) formation between HUT-78/HIV-1 or HUT-78/HIV-2 cells and Molt/4 (clone 8) cells by 50%.

[b] Concentration required to inhibit MT-4 cell viability by 50%.

[c] Minimal cytotoxic concentration, or minimal concentration resulting in a microscopically visible alteration of normal cell morphology.

[d] Data for dextran sulfate (MW 5000).

[e] Data taken from Balzarini et al. (1991).

GalNAc)- and Neu5Ac(α-2,6-Gal/GalNAc)-specific plant lectins were inhibitory at subtoxic concentrations. Of the $(GlcNAc)_n$-specific lectins, only UDA ($n = 1$) was able to suppress syncytium formation in the co-cultivation assay at a 50% effective concentration of 1–2 μg/ml, that is at a concentration 25-fold lower than the EC_{50} of DS-5000. At 5–10 μg/ml, UDA completely blocked syncytium formation (data not shown).

TABLE 3

Inhibitory effect of plant lectins on CMV-induced cytopathicity in HEL cells

Compound	EC_{50}[a] (μg/ml)	MCC[b] (μg/ml)	S.I.[c]
Man-specific plant lectins			
AUA	–	–	–
CA	9.1	100	19
EHA	15.7	100	15
LOA	1.1	100	90
GlcNAc-specific plant lectins			
WGA	0.36	2	5.5
$(GlcNAc)_n$-specific plant lectins			
STA	7.1	⩾ 100	⩾ 14
UDA	0.35	40	114
GalNAc/Gal-specific lectins			
APA	> 100	500	< 5
BDA	> 100	500	< 5
BPA	> 0.8	0.8	< 1
DBA	22	40	1.8
EHL	> 0.15	2	< 12
IRA	> 0.8	2	< 2.5
RPA	> 0.8	2	< 2.5
RSA	> 0.8	2	< 2.5
SBA	> 100	500	< 5
SNA II	> 20	100	< 5
Neu5Ac(α-2,3-Gal/GalNAc)-specific plant lectins			
MAA	–	2	–
Neu5Ac(α-2,6-Gal/GalNAc)-specific plant lectins			
SNA I	> 100	500	< 5
DHPG	0.8	⩾ 100	⩾ 125
DS-5000	0.35	> 100	> 286

[a] Concentration required to inhibit CMV-induced cytopathicity in HEL cells by 50%. Data represent the average values obtained for the Davis and AD-169 strains of CMV.

[b] Minimal cytotoxic concentration, or minimal concentration resulting in a microscopically visible alteration of normal cell morphology.

[c] Selectivity index or ratio MCC/EC_{50}.

The mannose-specific plant lectins LOA, CA and EHA were highly inhibitory to syncytium formation between HUT-78/HIV-1 or HUT-78/HIV-2 and Molt/4 cells (Table 2). In fact, they proved at least 30- to 60-fold more effective than DS-5000 under the same experimental conditions. No marked differences were noted in the efficacy of these plant lectins against syncytium formation with the persistently HIV-1- or HIV-2-infected HUT-78 cells. AUA, that was less efficient than the other Man-specific plant lectins in inhibiting HIV replication in MT-4 cells also proved ~10-fold less efficacious in inhibiting syncytium formation.

TABLE 4

Inhibitory effect of plant lectins on cytopathicity of various viruses (HSV-1, HSV-2, VV, VSV) in E_6SM cells

Compound	EC_{50}[a] (μg/ml)				MCC[b]
	HSV-1 (KOS)	HSV-2 (G)	VV	VSV	
Man-specific plant lectins					
AUA	–	–	–	–	–
CA	10	4	> 40	> 40	⩾ 100
EHA	40	7	> 40	> 40	⩾ 100
LOA	> 10	> 10	> 10	> 10	⩾ 40
GlcNAc-specific plant lectins					
WGA	> 10	7	> 10	> 10	40
$(GlcNAc)_n$-specific plant lectins					
STA	–	–	–	–	–
UDA	20	7	> 40	> 40	⩾ 100
GalNAc/Gal-specific lectins					
APA	–	–	–	–	–
BDA	> 10	> 10	> 10	> 10	40
BPA	0.07	0.2	0.7	> 1	4
DBA	2	7	> 10	> 40	⩾ 40
EHL	0.1	0.2	> 1	> 4	⩾ 4
IRA	0.07	0.7	> 1	> 4	⩾ 4
RPA	> 10	> 10	> 10	> 10	40
RSA	0.4	0.7	> 4	> 10	⩾ 10
SBA	–	–	–	–	–
SNA II	20	20	> 100	> 100	⩾ 200
Neu5Ac(α-2,3-Gal/GalNAc)-specific plant lectins					
MAA	0.4	0.7	> 1	> 1	⩾ 4
Neu5Ac(α-2,6-Gal/GalNAc)-specific plant lectins					
SNA I	–	–	–	–	–
DS-5000[c]	7.0	5.3	20	11	> 500

[a,b] As explained in footnotes a and b to Table 3.
[c] Data taken from Schols et al. (1990).

Influence of UDA, EHA and DS-5000 on binding of HIV-1 to MT-4 cells

When HIV-1 particles were preincubated with UDA, EHA or DS-5000 and then exposed to MT-4 cells under continuous presence of the test compounds, striking differences were observed between UDA and EHA on the one hand and DS-5000 on the other hand. At 25 μg/ml, DS-5000 completely blocked HIV-1 adsorption to MT-4 cells (compare in Fig. 1, panel D with panel A). In contrast, EHA only had a marginal effect (7% inhibition) on virus adsorption at a concentration (100 μg/ml) which was 200- to 2000-fold higher than the compound concentration required to inhibit HIV-1-induced cytopathicity in MT-4 cells and syncytium formation between persistently HIV-1- or HIV-2-infected HUT-78 and uninfected Molt/4 cells (Fig. 1, panel C). UDA had no

TABLE 5

Effect of temperature and presence or absence of the test compounds during the virus adsorption, virus–cell fusion or endocytosis process on their anti-RSV and influenza A virus activity

Compound	EC_{50}[a] RSV (μg/ml)		EC_{50} influenza A virus (μg/ml)	
	4°C, 1 h	35°C, 5 days	4°C, 1 h	35°C, 5 days
DS-5000	0.62	0.80	> 100	20
LOA	> 20	1.8[a]	> 20	1.8[a]
UDA	> 20	9.0[a]	> 20	1.8[a]

[a] 50% Effective concentration or compound concentration required to inhibit virus-induced plaque formation by 50%.

effect at all on HIV-1 binding to MT-4 cells (Fig. 1, panel D).

To reveal if HIV-1gp120 may bind directly to the cell surface through lectins, we examined virus binding to CD_4-negative human HSB-2 cells in the presence of 100 μg LOA/ml. No virus-bound cells could be detected according to the same methodology as used for the examination of HIV-1 binding to MT-4 cells (data not shown).

Interference of dextran sulfate and plant lectins with binding of anti-gp120 MAb to HUT-78/HIV-1-infected cells

It has been shown by Skinner and co-workers (1988) that the anti-gp120 MAb (NEA 9284) has no marked anti-HIV effect at 100 μg/ml. Therefore, under our experimental conditions (1 μg anti-gp120 MAb/ml), the MAb had no effect on the virus-induced cytopathicity per se. A moderate inhibition of anti-gp120 MAb (1 μg/ml) binding was observed when HUT-78/HIV-1 cells were preincubated with UDA at 20 μg/ml (55% inhibition), while a slightly more pronounced inhibitory effect was noted with EHA at 20 μg/ml and LOA at 40 μg/ml (73% and 79% inhibition, respectively). The inhibition was clearly concentration-dependent, and disappeared faster with UDA (13% and 0% inhibition at 4 and 0.8 μg/ml, respectively) than EHA (62 and 44% inhibition at 4 and 0.8 μg/ml, respectively) (data not shown). Under similar experimental conditions, DS-5000 completely inhibited anti-gp120 MAb binding to HUT-78/HIV-1-infected cells (by 99%) at a concentration of 25 μg/ml.

Effect of the temperature and presence or absence of the test compounds during the virus adsorption period on the anti-HIV-1 activity of plant lectins and dextran sulfate

MT-4 cells were exposed to a concentrated amount of HIV-1 at either 4°C or 37°C for 2 h in the presence or absence of the test compounds, and further incubated at 37°C for 2 additional days. HIV-1-specific antigen expression on the MT-4 cell membrane was examined by cytofluorometry (FACSTAR). When the MT-4 cells had been exposed to HIV-1 at 4°C for 2 h (in the presence of the compounds) prior to subsequent incubation of the cells at 37°C, a marked inhibition of virus-specific antigen expression was afforded by the plant

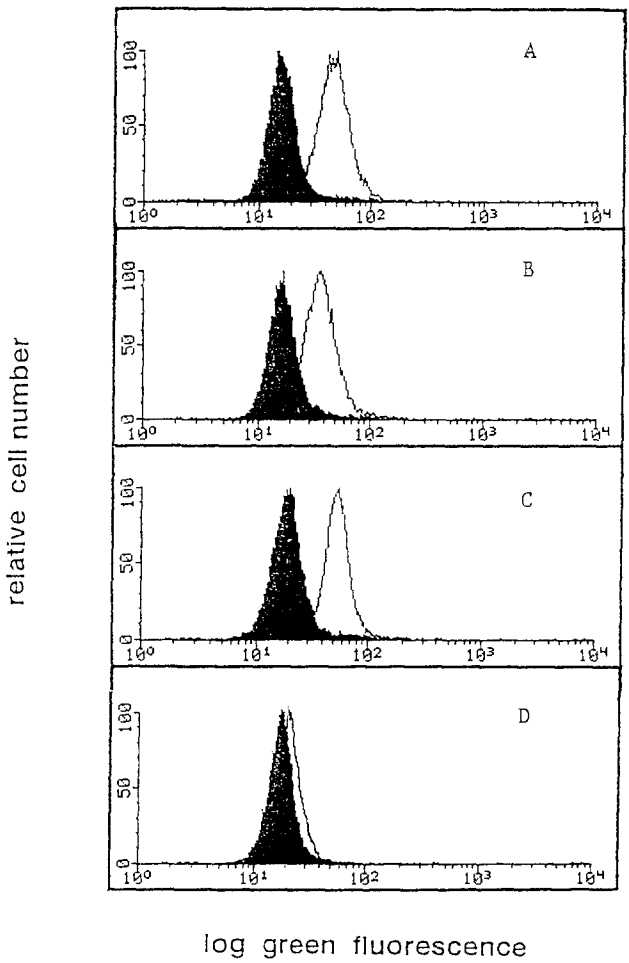

Fig. 1. Effect of EHA, UDA and DS-5000 on HIV-1 binding to MT-4 cells. The left solid histograms represent non-specific fluorescence in the absence of test compound (panel A) or in the presence of EHA (100 μg/ml) (panel B), UDA (100 μg/ml) (panel C), or DS-5000 (25 μg/ml) (panel D). The right open histograms represent the HIV-1-specific fluorescence in the absence of test compound (panel A) or in the presence of EHA (100 μg/ml) (panel B), UDA (100 μg/ml) (panel C) or DS-5000 (25 μg/ml) (panel D).

lectins LOA, EHA, UDA and by DS-5000 [EC_{50} ~0.01 μg/ml (for LOA and EHA) and ~0.5 μg/ml (for UDA) and ~0.01 μg/ml for DS-5000] (data not shown). When addition of the test compounds was delayed till after the 2-h adsorption period at 4°C, virtually no decrease in the inhibitory effects of the plant lectins on HIV-1 antigen expression was observed, whereas DS-5000 became markedly less (~10-fold) inhibitory under these conditions.

If the virus adsorption was allowed at 37°C (in the presence of the test compounds), virtually no differences were noted in the antiviral effects of the lectins and DS-5000, as compared to their effects at 4°C. However, whereas the absence of the test compounds during the adsorption period did not diminish

the antiviral effects of LOA, EHA and UDA, it markedly decreased the antiviral effect of DS-5000 (EC_{50}: ~ 0.02 μg/ml in the presence and EC_{50}: ~0.4 μg/ml in the absence of DS-5000) (data not shown).

Effect of the temperature and presence or absence of the test compounds during the virus adsorption period on the anti-RSV and anti-influenza A activity of plant lectins and dextran sulfate

Confluent HeLa cell cultures were exposed to RSV at 4°C for 1 h in the presence or absence of DS-5000, LOA or UDA. After aspiration of the culture medium, cells were incubated with warm medium with or without test compound to allow the virus to fuse with the cell membrane and further continue its infection cycle. Plaque formation was recorded after 5 days of incubation at 35°C (Table 5). When the test compounds were only present during the 1-h incubation period at 4°C, potent anti-RSV activity (EC_{50}: 0.62 μg/ml) was recorded for DS-5000, while the plant lectins LOA and UDA were devoid of any anti-RSV activity (EC_{50}: > 20 μg/ml) (Table 5). In contrast, when the test compounds were added after the initial 4°C-incubation periods, dextran sulfate, as well as LOA and UDA, proved markedly effective (EC_{50}: 0.80, 1.8 and 9.0 μg/ml, respectively). When the test compounds were added to influenza A virus-infected MDCK cells under identical incubation conditions as described for RSV, LOA, UDA and DS-5000 were only antivirally effective when continuously present after the adsorption period (EC_{50}: 20, 1.8 and 1.8 μg/ml, respectively) (Table 5).

Discussion

The antiviral activity spectrum of the plant lectins varies considerably, depending on the nature of their sugar specificity (i.e. mannose, *N*-acetylglucosamine, *N*-acetylgalactosamine). As a rule, the mannose-specific plant lectins are highly effective against lentiviruses (i.e. HIV-1, HIV-2, SIV), also markedly effective against CMV, but moderately active against HSV-1 or HSV-2. Only one *N*-acetylglucosamine-specific plant lectin (UDA) (out of 3 tested plant lectins with similar specificity) proved effective against lentiviruses, CMV, HSV-1 and HSV-2. None of the 12 *N*-acetylgalactosamine-specific plant lectins proved effective against HIV-1, HIV-2 or CMV, whereas several of them (i.e. BPA, DBA, EHL, RSV, IRA, MAA and SNA-II) proved effective against HSV-1 and HSV-2 at concentrations below their toxicity threshold. These differential antiviral specificities clearly reflect differences in the sugar specificity of the lectins, but, in addition, they may also be determined by other factors which are as yet poorly understood. The configuration of the sugar oligomers [i.e. the nature and type of the interglycosidic linkage (α or β; 1-3 or 1-6 binding)] and the presence of other sugar derivatives attached to the determinant sugar part may play an important role in the antiviral specificity demonstrated by the substances.

The two novel mannose-specific plant lectins CA and EHA are highly effective against HIV-1 and HIV-2, and rank among the most specific and potent plant lectins described so far. Their selectivity (ratio CC_{50}/EC_{50}) amounts up to 700–1500, and thus exceeds that of the previously described potent anti-HIV agent LOA by almost one order of magnitude. The effectiveness of mannose-specific plant lectins against HIV seems to be related to the presence in HIV-1 gp120 of more than 20 *N*-glycosylation sites which are extensively glycosylated, particularly with mannosidic oligosaccharides (Allan et al., 1985). These carbohydrate moieties consist of $(Man)_9GlcNac_2$ and are not trimmed by cellular mannosidases (Weiler et al., 1991). Also, our observation, that at least one GlcNac-specific (i.e. UDA), but none of the GalNac-specific lectins, is effective against HIV, is in agreement with the nature of the sugars present in the gp120 molecule.

The mannose-specific CA, EHA and LOA and GlcNAc-specific UDA also proved very effective in inhibiting syncytium formation between persistently infected HUT-78/HIV-1 or HUT-78/HIV-2 cells and uninfected $CD4^+$ Molt/4 (clone 8) cells. In this respect, the plant lectins were 25- to 100-fold more efficient against syncytium formation than dextran sulfate (DS-5000), a well-known inhibitor of HIV adsorption. In comparison with DS-5000, the protective effects of the plant lectins on giant cell formation between persistently infected HUT-78 and Molt/4 (clone 8) cells is much more pronounced than could be expected from their antiviral effects in primarily infected MT-4 cells. Also, the plant lectins interfere much less efficiently with anti-gp120 mAb binding to HUT-78/HIV-1 cells than DS-5000 does. LOA does not even interfere with anti-CD4 mAb binding to CD4 (Balzarini et al., 1991). Further, in striking contrast with DS-5000, the mannose-specific plant lectins LOA, CA and EHA as well as the GlcNAc-specific UDA do not inhibit HIV-1 binding to the target MT-4 cells at concentrations that are at least 3 orders of magnitude higher than their antivirally active concentrations. These observations let us conclude that, unlike the sulfated polysaccharides, the plant lectins do not significantly interfere with HIV adsorption to its target cells, but act at a subsequent step in the viral replicative cycle (i.e. viral fusion).

An additional argument for the fusion step as target in the antiviral activity of the plant lectins is based on our observations that absence of the plant lectins during the virus adsorption period does not alter their capacity to inhibit viral antigen expression, whereas, under these conditions DS-5000 becomes markedly less effective. These observations are also in agreement with the data reported by Matsui et al. (1990), who found that succinylated ConA inhibited HIV infection even when added after viral binding to cells, and our additional findings that DS-5000 is fully active against HIV-1 even when present only during the adsorption phase, while under the same conditions, the plant lectins LOA and UDA lose 95% of their antiviral activity (data not shown). Also, our antiviral data obtained for LOA and UDA against RSV and influenza A virus clearly provide evidence that the plant lectins exert their anti-RSV and anti-influenza A virus effect by interfering with a process

distinguished from and subsequent to the virus adsorption event. We could also prove that DS-5000, while interfering with the initial adsorption process during RSV infection, exerts its anti-influenza A virus activity – like the plant lectins – at a postadsorption process (Table 5).

Our observations that the plant lectins interfere less efficiently with anti-gp120 mAb binding to gp120-expressing HUT-78/HIV-1 cells than could be expected from their potent inhibitory effect on syncytium formation between HUT-78/HIV-1 and Molt/4 (clone 8) cells, suggest that the plant lectins may bind to molecules other than gp120. In fact, following binding of the viral gp120 to the cellular CD4 receptor during the virus adsorption process, the highly lipophilic transmembrane glycoprotein (gp41) normally hidden beneath gp120 may be exposed so as to generate the fusion of the virus particle with the target cell as well as fusion of the gp120 expressing cells (i.e. HUT-78/HIV) with uninfected $CD4^+$ cells (i.e. Molt/4 (clone 8)).

The data presented here and previously (Balzarini et al., 1991) and those presented by Matsui et al., 1990 suggest that the anti-HIV activity of the plant lectins is directed against the virion-cell fusion step. Also, it has recently been suggested by Jansen and co-workers (1991) that several neoglycoproteins are inhibitory to HIV replication by interfering with a post-HIV binding event, presumably fusion of the virus with its target cells. The exact mode of action of the lectins remains to be elucidated. Such studies appear to be justified as they will increase our insight in the mechanism of virus infection and virus–cell fusion.

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