Novel Polysulfated Galactose-Derivatized Dendrimers as Binding Antagonists of Human Immunodeficiency Virus Type 1 Infection

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Evidence indicates that galactosyl ceramide (GalCer) and its 3'-sulfated derivative, sulfatide (SGalCer), may act as alternate coreceptors for human immunodeficiency virus type 1 (HIV-1) in CD4⁺ cells. Glycosphingolipids (GSLs) may also be necessary for fusion of HIV-1 and host cell membranes. Using an enzyme-linked immunosorbent assay to determine which GSL was the best ligand for both recombinant and virus-associated gp120, we found that SGalCer was the best ligand for each rgp120 and HIV-1 isolate tested. Therefore, novel multivalent glycodendrimers, which mimic the carbohydrate clustering reportedly found in lipid rafts, were synthesized based on the carbohydrate moiety of SGalCer. Here we describe the synthesis of a polysulfated galactose functionalized, fifth generation DAB dendrimer (PS Gal 64mer), containing on average two sulfate groups per galactose residue. Its ability to inhibit HIV-1 infection of cultured indicator cells was compared to that of dextran sulfate (DxS), a known, potent, binding inhibitor of HIV-1. The results indicate that the PS Gal 64mer inhibited infection by the HIV-1 isolates tested as well as DxS.

Entry of human immunodeficiency virus type 1 (HIV-1) into susceptible cells is a complex process mediated by the gp120-gp41 viral envelope glycoprotein complexes (49). In CD4-expressing cells, trimerized gp120-gp41 complexes (5, 49) interact with CD4 molecules, inducing a conformational change in gp120 (21, 49) that allows for its subsequent interaction with a chemokine coreceptor (53): CXCR4 for T-cell-tropic and CCR5 for macrophage-tropic viral strains, designated X4 and R5 viruses, respectively (1). The interaction of gp120 with a chemokine coreceptor promotes the exposure and subsequent insertion of the hydrophobic gp41 fusion peptide into the host cell membrane (4, 5, 7, 48, 49). This insertion is thought to promote the assembly of a molecular scaffold, or fusion pore, that enables the injection of the HIV-1 nucleocapsid into the host cell (21, 31, 34). In non-CD4-expressing neuronal (18), vaginal (11), and colonic cells (50), glycosphingolipids (GSLs) have been implicated as alternative receptors for the docking of HIV-1 virions and migration to the chemokine coreceptors (2, 8, 14, 18, 50).

Cell surface GSL rafts are naturally involved in membrane trafficking, cell adhesion, cell morphogenesis, and signal transduction (12, 24, 39, 40). In addition to their natural physiological roles, lipid rafts are exploited by a variety of pathogens (bacteria and viruses) and toxins for cellular entry (10, 16). As regards HIV-1 infection, receptors CD4 (30) and CCR5 (28)

have been shown to be colocalized within lipid rafts, suggesting that HIV entry preferentially occurs at lipid rafts (41). This hypothesis is supported by the observation that methyl- β -cyclodextrin-induced depletion of plasma membrane cholesterol (which is necessary for lipid raft formation) inhibited HIV-1 infection of CD4⁺ T cells by 95%, while not affecting the expression of the HIV-1 receptors (36). Correspondingly, PPMP (1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol)-induced depletion of GSL biosynthesis—also necessary for lipid raft formation—in CD4⁺ human osteosarcoma cells inhibited infection by a variety of HIV-1 isolates (19). These observations, together with those of several other studies (28, 30, 37, 41, 42), have led to the hypothesis that lipid rafts represent binding and entry sites for HIV-1 (36). It is believed that since membrane fusion is such a cooperative process—requiring multiple copies of several different receptors—concentrating these receptors in lipid rafts would bring them into close proximity, increasing the probability of forming a fusion pore (36). Therefore, since GSL rafts may act as the glue that holds together the components needed for HIV-1 entry, it is logical to target GSL rafts as potential sites for therapeutic intervention. We hypothesized that binding antagonists of HIV-1 could be developed by synthesizing multivalent, GSL-lipid raft-mimicking glycoconjugates. If successful, these conjugates would provide an approach for the development of new binding antagonists that could inhibit viral transmission and potentially reduce viral load.

In this study, we characterized the adherence of several viral isolates with different cellular tropisms [HIV-1 IIIB (X4), MN (X4), and Ba-L (R5)], and the corresponding rgp120s (gp120 IIIB, MN, and Ba-L) to GSLs immobilized on polyvinyl chloride wells. The results of the binding studies led us to hypothesize that a multivalent sulfated galactose-derivatized dendrimer might be an effective antagonist of HIV-1 binding. Here

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we describe the synthesis of a polysulfated galactose functionalized glycodendrimer (PS Gal 64mer) that contained on average two sulfate groups per galactose residue. The ability of the PS Gal 64mer to inhibit HIV-1 infection of cultured indicator cell lines was compared to that of dextran sulfate (DxS), a known potent inhibitor of HIV-1 (X4) infectivity. The results indicated that (i) of the GSLs tested, immobilized SGalCer was the best ligand for all of the virions and rgp120s studied; (ii) monosulfated galactose saccharides were unable to inhibit rgp120 adherence to immobilized sulfatide; and (iii) the PS Gal 64mer was as effective an inhibitor of HIV-1 infectivity or better than DxS for the isolates tested.

MATERIALS AND METHODS

Purified GSLs were purchased from Matreya, Inc. (Pleasant Gap, Pa.). Recombinant gp120 IIIB was bought from ImmunoDiagnostics (Buffalo, N.Y.), rgp120 MN was obtained from ImmunoDiagnostics through the National Institutes of Health (NIH) AIDS Reagent Program, and rgp120 Ba-L was a generous gift from Raymond Sweet (SmithKline Beecham, King of Prussia, Pa.). Human polyclonal anti-HIV immunoglobulin G (IgG) antibody was from the AIDS Reagent Program. Directly pelleted HIV-1 viruses IIIB, MN, Ba-L, and 89.6 were obtained from ABI (Columbia, Md.). Ninety-six-well polyvinyl chloride microplates (Falcon; Becton Dickinson, Franklin Lakes, N.J.) were purchased from Fisher Scientific (Pittsburg, Pa.). Polypropylenimine tetrahexacontaamine dendrimer, generation 5 (DAB-Am-64), was from Aldrich (Milwaukee, Wis.). DxS (8 kDa), DxS (Dextralip 50, lot 71K1378), chondroitin sulfate (ChS) (45 kDa), glucose-3-sulfate, galactose-6-sulfate, galactose-4-sulfate, and sialyllactose were from Sigma (St. Louis, Mo.). Galactose-3-sulfate was isolated from SGalCer as previously described (29, 52).

ELISA for monitoring rgp120 binding to immobilized GSLs. Lipid-coated microplate wells were prepared by adding 1.2 nmol (equivalent to 1 μ g of SGalCer) of GSL in 50 μ l of methanol/chloroform (9:1 [vol/vol]) per well and drying overnight. Each measurement was done in quadruplicate, and each experiment was repeated at least once. Nonspecific protein binding sites were blocked by incubating wells with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) (wt/vol) for 1 h at 37°C, prior to the addition of 0.4 μ g of either rgp120 IIIB, MN, or Ba-L in 100 μ l of 0.1% BSA in PBS to the appropriate wells. Nonspecific rgp120 adherence was monitored using protein-only controls (wells that were treated with methanol-chloroform only with no GSLs). After incubating for 1 h at 37°C, wells were washed six times with PBS to remove nonadherent rgp120. Bound rgp120 was visualized by using an indirect immunoassay in which 100 μ l of a human polyclonal anti-HIV IgG antibody, diluted (1:1,000) using a solution of 2% BSA in PBS, was added to each well and incubated for 1 h at 37°C. Nonbound antibody was removed by washing the wells three times with PBS. Wells were then incubated for 1 h at 37°C with 100 μ l of a solution of horseradish peroxidase-conjugated, goat anti-human IgG antibody (Sigma) diluted (1:10,000) in a solution of 2% BSA in PBS. Nonspecific cross-reactivity between the lipids and antibodies was monitored using lipid-only controls (wells that were treated with antibodies only with no rgp120). After washing the wells three times to remove unbound secondary antibody, bound horseradish peroxidase was monitored by measuring the oxidation of *O*-phenylenediamine (*O*-pd). The reaction was carried out in 100 μ l of a solution of 0.1 M sodium citrate in 0.02 M Na₂HPO₄ buffer (pH 5.0), containing 0.04% *O*-pd and 0.003% H₂O₂. After 25 min in the dark at room temperature, the reaction was stopped by the addition of 25 μ l of 0.2 N H₂SO₄. Optical density (OD) was read at 490 nm with a reference filter of 630 nm using a DYNEX MRX II automated microplate reader. Data were analyzed using Revelation MRX software (Dynex, Chantilly, Va.). Specific binding was determined by subtracting the average of protein-only and lipid-only control wells from the average of the experimental wells treated with both protein and lipid. Since SGalCer was found to be adhered to best by each isolate tested, the results were standardized to SGalCer as 100% binding. Standardization of the results to binding to SGalCer, measured in each experiment, eliminated variation due to differences in OD values obtained when experiments were done on different days.

A modified quantitative resorcinol-sulfuric acid colorimetric assay of neutral sugars was used to determine whether equivalent amounts of the different GSLs were immobilized (32). Briefly, 1 μ g of each lipid was added in methanol to each of four microtiter wells. Once dry, the lipid-coated wells were incubated with 200 μ l of PBS for 1 h at 37°C to simulate the blocking period of the enzyme-linked

immunosorbent assay (ELISA). After the incubation period, the wells were washed six times and 20 μ l of resorcinol reagent (6 mg/ml in distilled water) was added, followed by 100 μ l of 75% sulfuric acid. The microtiter plate was shaken and incubated in an oven at 80°C for 1 h. Absorbance was then read at 450 nm. The results indicated that similar amounts of each lipid remained in the wells after the initial blocking step (data not shown).

ELISA for monitoring HIV-1 binding to immobilized GSLs. Adherence of HIV-1 isolates to immobilized GSLs was monitored using the ELISA for gp120 described above, with the exception that 2.0 N H₂SO₄ was used to stop the *O*-pd reaction, followed by the addition of 25 μ l of 5% Triton X-100 to each well to kill live virus. The ideal concentration of virions needed to observe binding was determined by monitoring the adherence of increasing amounts of HIV-1 IIIB to SGalCer-coated wells. The amount of virus used in subsequent experiments fell within the linear range of binding and corresponded to a p24 concentration of approximately 3×10^5 pg/ml. To rule out the possibility that the HIV-1 ELISA was detecting the adherence of gp120 sheared from the virions, the presence of virions was determined by using the p24 ELISA capture assay. SGalCer-coated wells and control, nonlipid-coated wells were incubated with HIV-1 IIIB, washed extensively to remove unbound virions and protein, and treated with Triton X-100 to lyse adherent virions prior to measuring p24 content using the p24 antigen capture assay (Dupont-NEN). The results showed that as the amount of HIV-1 IIIB added per well increased, so did the concentration of p24 detected in SGalCer-coated wells (data not shown). Therefore, viral adherence, not shed gp120, was detected.

ELISA comparison of the different viral isolates binding to SGalCer. In order to compare the binding of the different viral isolates to SGalCer, viral stocks were diluted so that they contained approximately equivalent amounts of p24 as determined using the p24 antigen capture assay (Dupont-NEN). Each diluted stock (100 μ l) was then added to SGalCer-coated wells and incubated for 1 h at 37°C. Adherence was monitored using the indirect immunoassay for HIV-1 gp120 described above. Each of these experiments was done in quadruplicate, and each was repeated at least once. Absorbances from the viral binding to SGalCer wells were standardized to a p24 concentration of 0.15 μ g/ml.

ELISA binding inhibition assays. The rgp120 ELISA assay described above was used to study the ability of different GSLs and saccharides to inhibit the adherence of rgp120 to immobilized sulfatide. Increasing concentrations of SGalCer, GalCer, GM3, free GSL saccharides, DxS (8 kDa), or ChS (50 kDa) were preincubated with rgp120 IIIB in a solution of 0.1% BSA in PBS for 1 h at 37°C prior to addition of the mixture to SGalCer-coated wells. Percent inhibition was calculated as $100(B - B_i/B)$, where B is the binding of rgp120 IIIB to SGalCer and B_i is the binding to SGalCer in the presence of inhibitor. Fifty percent inhibitory concentrations (IC₅₀) were calculated by plotting the percent inhibition versus the log of the concentration of the inhibitor used and then determining the concentration at which adherence was reduced by 50%.

Synthesis of polysulfated galactose functionalized, generation 5.0 glycodendrimers (PS Gal 64mer). Generation 5.0 DAB dendrimers (64 terminal amines, see Fig. 3B) were functionalized with 3-(galactosylthio)propionic acid residues (25a) and are referred to here as Gal 64mer (Fig. 3C). Gal 64mer (60 mg), containing on average 44 galactose residues with an M_w of 18,261, was taken up in 2 ml of dry dimethyl sulfoxide. Sulfur trioxide pyridine complex at 2 eq per hydroxyl was then added, and the mixture was stirred for 5 h at 37°C (51). After the incubation period, pyridine was added to the mixture to yield a 1.0 M pyridine mixture that was then applied directly to a Biogel P2 column and eluted with 1.0 M pyridine acetate buffer (pH 5.0). Thin-layer chromatography plates were spotted with aliquots of fractions eluted in the void volume, and the presence of carbohydrate-containing compounds was identified by charring with 5% H₂SO₄ in ethanol. Carbohydrate-containing fractions were pooled and dried several times from toluene by rotary evaporation. The resulting film was taken up in 2 ml of H₂O and passed through a small amount of Amberlite (Na⁺; 100 to 200 mesh) resin to convert the SO₃⁻ pyridine salt to the Na⁺ salt form. The resin was washed with H₂O, the eluate was dried by rotary evaporation, and the resulting film was lyophilized. Matrix-assisted laser desorption/ionization-time of flight mass spectrometry analysis of the product revealed that the resulting compound had a polydispersity of 1.02 and an M_w of 26,789, which corresponded to an average of 84 sulfate groups incorporated when the M_w of the galactosylated dendrimer was subtracted from that of the sulfated product and the difference was divided by 102 Da (the mass of the sodium salt of one added sulfate group).

Cell culture. U373-MAGI-CXCR4 and U373-MAGI-CCR5 cells (catalog no. 3596 and 3597, respectively) were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, from Michael Emerman and Adam Geballe (47). U373-MAGI cells were propagated in selection medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with

10% fetal calf serum (FCS), 0.4-mg/ml L-glutamine, 0.08-mg/ml each penicillin and streptomycin, 0.05% sodium bicarbonate, 0.2-mg/ml G418, 0.1-mg/ml hygromycin B, and 1.0- μ g/ml puromycin. Culture medium for virus inhibition assays consisted of DMEM with 10% FCS and 0.05% sodium bicarbonate.

Viral inhibition assays. Cells were plated in 96-well tissue culture plates at 1.5×10^4 cells per well in selection medium. One day after plating the cells, concentrated cell-free viral preparations of HIV-1 IIIB (X4), NL4-3 (X4), and 89.6 (X4/R5) were diluted 1:200, 1:100, and 1:10, respectively, with culture medium. Glycodendrimer and DxS (50 kDa) 2-mg/ml stocks were made up in culture medium and serially diluted with culture medium in sterile V-bottom 96-well microtiter plates to a final volume of 30 μ l per well. This was followed by the addition of 30 μ l of diluted virus and incubation at 37°C for ~10 min. The selection medium was removed from the plated cells, and 50 μ l of the inhibitor-virus mixtures was then added to each well of the plated cells. Virus was allowed to absorb to cells for 2 h at 37°C in an atmosphere of 95% air–5% CO₂. After the absorption period, 200 μ l of culture medium was added to the 50 μ l of inhibitor-virus mixture in each well and the microtiter plates were incubated for 40 to 48 h at 37°C in an atmosphere of 95% air–5% CO₂. Cells treated with culture medium served as negative controls, and cells treated with virus with no inhibitor were positive controls. All measurements were done in quadruplicate.

After the 48-h incubation, culture medium was removed from the wells, the cells were washed with 200 μ l of PBS, and β -galactosidase activity was measured using the Galacto-Star β -galactosidase reporter gene assay system from Applied Biosystems (Foster City, Calif.), according to the manufacturer's instructions. Briefly, after the cells were washed with PBS, 10 μ l of the provided lysis solution was added to each well, and the plate was incubated for 10 min at 37°C. The Galacto-Star substrate was diluted 1:50, and 100 μ l was added to each well containing cell lysates. Well contents were mixed, and 90 μ l was removed and added to a 96-well opaque luminometer plate. One hour after the Galacto-Star substrate was added, the luminescence was counted on a luminometer. Percent inhibition of each compound was determined, and the effective concentration that inhibited 50% of viral infectivity (EC₅₀) was determined by plotting the percent inhibition by the log of the concentration of each of the compounds tested.

Cytotoxicity assay. The effect of the glycodendrimers and DxS on cell viability was determined using the CellTiter 96 AQueous cell proliferation assay (Promega, Madison, Wis.). In this viability assay, mitochondrial dehydrogenases in metabolically active cells catalyze the conversion of MTS [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt)] into a formazan product. The absorbance of formazan is read at 490 nm and is directly proportional to the number of viable cells. Assays were performed according to the manufacturer's instructions.

Briefly, replicate 96-well plates of each of the HIV infection inhibition assay plates were set up and treated identically, with the exception that no virus was added to the cells. After the 48-h incubation period, the cell culture medium was removed, the cells were washed with PBS, and 100 μ l of culture medium was added to each well. The MTS reagent was mixed with an electron coupling reagent (phenazine methosulfate) according to the instructions, and 25 μ l of the mixture was added to the cells. The 96-well replicate plates were incubated at 37°C in an atmosphere of 95% air–5% CO₂. After 3 h, samples were removed and the A₄₉₀ was read on an ELISA plate reader. Cell viability indices were calculated by dividing the average absorbance of the nontreated cells (negative control wells) into the average absorbance obtained for each concentration of each of the compounds tested. In this way, nontreated cells had a cell viability index of 1.0 while that of cells exposed to cytotoxic compounds would be less than 1.0.

RESULTS

Identification of GSLs adhered to by several rgp120s and their corresponding viral isolates. Several different HIV-1 isolates with different cellular tropisms [HIV-1 IIIB (X4), MN (X4), and Ba-L (R5)], and the corresponding rgp120s (gp120 IIIB, MN, and Ba-L), were tested for their ability to bind to GSLs immobilized on microtiter wells by using the ELISA described in Materials and Methods. SGalCer (see Table 1 for the carbohydrate composition of the GSLs discussed) was the best ligand for all of the rgp120s and viruses studied, regardless of their cellular tropism (Fig. 1A to C). The rgp120s also bound to the negatively charged gangliosides GM3, GD3, and GM2, whereas the virions did not. At the concentration of GSLs tested (1.2 nmol, ~1 μ g of SGalCer), none of the rgp120s or

TABLE 1. Composition of the saccharide moieties of GSLs studied

Common name ^a	Structure
Galactosyl ceramide	
(GalCer)	Gal- β 1-Cer
GalCer-NFA	Gal- β 1-Cer-nonhydroxylated fatty acid
GalCer-HFA	Gal- β 1-Cer-hydroxylated fatty acid
Lactosyl ceramide	
(LacCer)	Gal(β 1-4)Glc β 1-Cer
Sulfatide (SGalCer)	Gal-3-SO ₃ β 1-Cer
Ceramide trihexoside	
(CTH)	Gal(α 1-4)Gal(β 1-4)Glc β 1-Cer-hydroxylated fatty acid (mostly)
Gb3	Gal(α 1-4)Gal(β 1-4)Glc β 1-Cer-nonhydroxylated fatty acid
GM3	Sialic acid (α 2-3)Gal(β 1-4)Glc β 1-Cer
GM2	GalNAc(β 1-4)[sialic acid(α 2-3)]Gal(β 1-4)Glc β 1-Cer
GD3	Sialic acid(α 2-8)[sialic acid(α 2-3)]Gal(β 1-4)Glc β 1-Cer

^a GSLs are listed in order of increasing polarity as determined by thin-layer chromatography in CHCl₃–CH₃OH–0.03% CaCl₂ (30:17.5:4 [vol/vol/vol]) solvent.

HIV-1 virions bound to GalCer—the implicated CD4⁺ neuronal cell receptor for gp120—or Gb3—the implicated CD4⁺ cell fusion cofactor for HIV-1 infection. Interestingly, rgp120 binding to GalCer was observed when the amount of GalCer added to each well was increased fivefold; however, the binding observed at the higher GalCer concentrations was still less than half that observed to 1 μ g of SGalCer (Fig. 1D). The observed preference of gp120 for SGalCer prompted experiments to determine whether several different HIV-1 isolates with different cellular tropisms would behave analogously. The concentration of HIV-1 IIIB (X4), MN (X4), Ba-L (R5), and 89.6 (X4/R5) used was based on the p24 content of each virus sample, and binding was determined by using the indirect immunoassay (Fig. 1E). The results of this study indicate that, for the isolates tested, cellular tropism had no effect on HIV-1 binding to SGalCer.

Inhibition of rgp120 adherence to immobilized sulfatide. ELISA binding inhibition studies were done to determine whether rgp120 binding to SGalCer was specific or due to nonspecific sulfate interactions and to ascertain which portion of SGalCer (the saccharide, the ceramide, or both) was required for binding. Therefore, the effectiveness of GSLs, polysulfated polysaccharides, and free saccharides at blocking the binding of rgp120 IIIB to immobilized SGalCer was determined. The results indicate that SGalCer was the most effective inhibitor of rgp120 IIIB binding to immobilized SGalCer (IC₅₀ of ~0.750 μ M). The next best inhibitor (IC₅₀ of 20 μ M) was the polysulfated polysaccharide DxS, which is known to have potent anti-HIV activity on X4 viral strains, followed by ganglioside GM3 (IC₅₀ of ~100 μ M). The polysulfated polysaccharide ChS showed only slight inhibition at the highest concentration tested, 100 μ M. GalCer and ceramide had no effect on rgp120 binding (Fig. 2A). Various free saccharides corresponding to the saccharide moieties of GM3 (sialyllactose) and SGalCer (galactose-3-sulfate), as well as other monosulfated hexoses (glucose-3-sulfate, galactose-4-sulfate, and galactose-6-sulfate), were not effective at blocking rgp120 binding to immobilized SGalCer at the concentrations tested (Fig. 2B). These observations indicate that the ceramide por-

FIG. 1. Identification of GSLs bound by rgp120 and by HIV-1. GSLs immobilized on 96-well microtiter plates were tested as ligands for HIV-1 IIIB, MN, and Ba-L and the corresponding rgp120s. For comparative purposes, binding to SGalCer was taken as 100%. (A) rgp120 IIIB and HIV-1 IIIB. (B) rgp120 MN and HIV-1 MN. (C) rgp120 Ba-L and HIV-1 Ba-L. (D) Comparison of rgp120 IIIB binding to 1 μ g of SGalCer to rgp120 IIIB binding to increasing concentrations (1, 2, 5, and 10 μ g) of GalCer. (The carbohydrate composition of each GSL is given in Table 1.) (E) Comparison of the binding of four different isolates of HIV-1 with different cellular tropisms [HIV-1 IIIB (X4), MN (X4), Ba-L (R5), and 89.6 (X4/R5)] to 1 μ g of SGalCer. Measurements were done in quadruplicate. Error bars represent the standard deviation with $n = 3$ or 4. Replicate experiments gave similar results.

tion was necessary, since that was the only difference between the galactosyl-3-sulfate and sulfatide used in this study. They also support the hypothesis that the ceramide portion might serve to present a cluster of saccharides for binding by the rgp120 or virus, rather than as part of the binding site.

Viral inhibition by multivalent GSL-derived glycoconjugates. Because SGalCer was found to be the best ligand for both recombinant and virus-associated gp120, multivalent glycodendrimers bearing sulfated galactose residues were synthe-

sized and tested for their ability to inhibit HIV-1 infection of cultured reporter cells as described in Materials and Methods. Figure 3 depicts the structures of SGalCer and the synthesized glycodendrimers. The results of the viral inhibition assays (Fig. 4) indicate that for the three HIV-1 isolates tested [(HIV-1 IIIB (X4), NL4-3 (X4), and 89.6 (X4/R5)], the novel PS Gal 64mer glycodendrimer was an inhibitor of HIV-1 infectivity as effective as or better than DxS, with nanomolar EC_{50} s. The results from the cytotoxicity studies indicate that neither the

FIG. 2. Inhibition of rgp120 IIIB binding to SGalCer. GSLs and polysulfated polysaccharides (A) or (B) free saccharides (B) were preincubated with rgp120 IIIB prior to overlaying the mixture on microtiter wells coated with $1 \mu\text{g}$ of SGalCer. Percent inhibitions were calculated, and IC_{50} s were determined as described in Materials and Methods. Measurements were done in quadruplicate. Error bars represent the standard deviation with $n = 3$ or 4. Replicate experiments gave similar results.

glycodendrimers nor DxS was cytotoxic to the cells at any of the concentrations tested, up to 3 mg/ml (Fig. 4D).

DISCUSSION

GSLs are used as receptors by a wide variety of pathogens including bacteria, fungi, and viruses (23). Soon after the discovery that CD4 was the primary receptor for HIV, it became apparent that an alternate mode of infection existed, since several CD4^- neuronal cell lines were found to be susceptible to infection by HIV (17, 27). In 1991, GalCer and its 3'-sulfated derivative, SGalCer, were reported to be the receptors that permitted HIV-1 infection in these cell types (2, 17). Subsequent studies showed that the interaction of gp120 with GalCer was of high affinity, having a dissociation constant (K_D) of 11.4 nM (2, 17), which is on the order of that reported for CD4 (K_D of 2 to 5 nM) (26). Other studies implicated GalCer,

SGalCer, and sulfated LacCer as possible coreceptors for HIV-1 infection of CD4^- neural, colonic, and vaginal cells, respectively (11, 18, 50). Also, ganglioside GM3 and ceramide trihexoside (Gb3) have been shown to associate with CD4 and CXCR4, respectively (13–15, 37, 38), and Gb3 has been implicated as being involved in fusion of viral and host cell membranes (38). In our efforts to develop binding antagonists of HIV-1 by synthesizing multivalent, lipid raft-mimicking glycoconjugates, we used an ELISA to determine which of the GSLs implicated in the literature as ligands or fusion cofactors for gp120 serves as the best ligand for both recombinant and virus-associated gp120.

The fact that recombinant gp120 does not always mimic its oligomeric, virus-associated counterpart (45) led us to compare the adherence of several rgp120s to that of the corresponding viral isolate (Fig. 1A to C). The results from these studies indicated that SGalCer was the most effective ligand

FIG. 3. Structures of (A) SGalCer; (B) a generation 5.0 DAB-Am dendrimer; (C) the galactose functionalized 5th generation DAB-Am dendrimer, Gal 64mer; and (D) the randomly polysulfated galactose functionalized 5th generation DAB-Am dendrimer, PS Gal 64mer, which contained on average ~ 2 sulfate groups per galactose residue.

for each of the rgp120s and HIV-1 virions tested, regardless of cellular tropism. Our observation that rgp120 adhered to GM3, while the HIV-1 virions adhered poorly, or not at all, to GM3 and Gb3 is interesting in light of their putative roles as fusion

cofactors (13–15). The results also showed that neither the rgp120s nor the virions adhered to GalCer when 1.2 nmol of lipid was added per well, despite its reported high-affinity interaction with gp120 and alleged importance in HIV-1 infec-

FIG. 4. Inhibition of HIV-1 infectivity and assessment of cytotoxicity. (A) Inhibition of HIV-1 IIIB infection of U373-MAGI-X4 cells by DxS and PS Gal 64mer. (B) Inhibition of HIV-1 NL4-3 infection of U373-MAGI-X4 cells by DxS and PS Gal 64mer. (C) Inhibition of HIV-1 89.6 infection of U373-MAGI-R5 cells by DxS and PS Gal 64mer. (D) Cytotoxicity of DxS and PS Gal 64mer on U373-MAGI-X4 cells. Measurements were done in quadruplicate. Error bars represent the standard deviation with $n = 3$ or 4. Replicate experiments with HIV-1 IIIB gave similar results.

tion of CD4⁺ cells. However, rgp120 adherence to GalCer was observed when the concentration of GalCer applied per well was increased 5- or 10-fold. Yet, the binding seen to wells coated with 10 μ g of GalCer was still less than that to wells coated with 1 μ g of SGalCer (Fig. 1D).

These results permit the hypothesis that the binding observed to immobilized SGalCer (Fig. 1) reflects a greater affinity of rgp120 for SGalCer than for the other GSLs tested. This interaction appears to be specific and not merely due to weak electrostatic interactions, since ganglioside GD3—containing four sugar residues and two negative charges, one on each of the sialic acid groups—was not bound to as well as SGalCer, which contains only one sugar residue and one negative charge. If the interaction with SGalCer was based on charge alone, it would be expected that the negatively charged, sialylated GSLs would be bound by rgp120 with equal or greater avidity. Since this was not observed, it can be concluded that SGalCer is the preferred GSL ligand for gp120. This apparent specificity was supported by ELISA binding inhibition studies (Fig. 2A and B) done to determine whether rgp120 adherence to SGalCer was specific or was due to nonspecific binding of the rgp120 to negatively charged ligands. The differences seen between SGalCer, GalCer, DxS, ChS, and GM3 in the ELISA binding inhibition assays indicate that the gp120 interaction with GSLs is not due solely to the presence of a negatively charged substituent.

Sulfated polysaccharides such as DxS, curdlan sulfate, pentosan sulfate, and heparin sulfate (Fig. 5) have long been

known to be inhibitors of HIV-1 X4 isolates. Binding of gp120 to these molecules varies depending on the viral isolate and may reflect the type of saccharide residues, as well as the degree and positions of the sulfation. This is evident in the fact that ChS and dermatan sulfate display little anti-HIV activity unless they are supersulfated (22). The inhibitory effects of these polysaccharides result from their interaction with the positively charged V3 loops on X4 gp120s, which hinders the interaction of the gp120 with its chemokine coreceptor (3, 20). DxS is made up of chains of repeating polysulfated glucose residues, containing approximately 2.3 sulfate groups per glucosyl residue (Fig. 5). In the ELISA binding inhibition assays (Fig. 2), DxS with a molecular mass of 8 kDa was used, which corresponds to approximately 62 sulfate groups per molecule. ChS, on the other hand, contains one sulfate group per repeating disaccharide unit. In the inhibition study, ChS with an average molecular mass of 45 kDa was used, corresponding to ~99 sulfate groups per molecule. Therefore, DxS and ChS contained 50- to 100-fold-more sulfate residues per molecule than SGalCer with one sulfate group. The molar percent inhibition differences (Fig. 2) seen between the sulfated compounds SGalCer, DxS, and ChS suggest a specific or preferential interaction between rgp120 and SGalCer, rather than simple nonspecific, negatively charged, sulfate interactions with the V3 loop of gp120, as reported in the literature (46). If the interaction of gp120 with SGalCer were based solely on nonspecific charge interactions with a sulfate group, then both DxS and ChS would be expected to be much more effective

FIG. 5. Structures of polysulfated polysaccharides.

inhibitors than SGalCer. Therefore, other factors must be involved in gp120 binding. The lack of binding to ChS agrees with the observation that ChS does not bind to gp120 (46).

From the results shown here and the data in the literature, it can be concluded that SGalCer is the best GSL ligand for gp120. It was surprising that gp120 bound so much better to SGalCer than to GalCer, since gp120 has been reported to bind to GalCer with a nanomolar affinity and GalCer is thought to be its receptor on CD4⁺ neuronal and colonic cells. However, a study by Fantini et al. (9) supports our observation. They found that the addition of SGalCer to the plasma membrane of CD4⁺ GalCer⁺ CXCR4⁺ HT-29 cells increased HIV-1 binding to the cells but inhibited infection. This was attributed to the gp120 binding so strongly to SGalCer that it competed out its interaction with CXCR4 (9). The affinity of gp120 for SGalCer provides a potential target for the development of HIV-1 binding antagonists.

In other instances where GSLs serve as receptors for pathogens, it is believed that the carbohydrate moiety of the GSL is the recognition site (6, 25). For example, studies of cholera toxin indicated that the ceramide moiety of GM1 was needed to present or position the sugars in such a way that they were available for binding by the pentavalent binding subunit (43, 44). In regard to HIV-1 adherence to GSLs, this question was partially addressed by comparing the ability of GSLs or their corresponding saccharide moieties to inhibit the adherence of rgp120 IIIB to immobilized SGalCer (Fig. 2A and B). The observation that the free saccharides tested were not effective inhibitors of rgp120 adherence to immobilized SGalCer, combined with the observation that gp120 did not bind to ceramide (Fig. 1A to C and 2A), indicates that the ceramide moiety is probably necessary for positional presentation, or clustering, of the saccharides such that they are available for binding.

Based on these findings, we decided to test the hypothesis

that galactose-3-sulfate functionalized dendrimers might mimic the carbohydrate clustering that would be present in a SGalCer-containing lipid raft. SGalCer was not considered as a potential antagonist for HIV-1 because it has been shown that when GSLs are added to cells in vitro, they can intercalate into the lipid bilayer of the plasma membrane, thereby becoming functional components of the membrane (33). It is this property of GSLs that led us to look for an alternative. Our success with using dendrimers derivatized with the saccharide portion of GM1 to inhibit the binding of cholera toxin to cells (43) led us to use that approach in these studies, the premise for both being that the ceramide portion of the GSL is what anchors it in the membrane and is not available for binding by the pathogen. Due to problems encountered in the synthesis of a 3-(β -D-3-sulfo-galactopyranosylthio)propionic acid functionalized dendrimer (25a), a polysulfated galactose functionalized dendrimer (PS Gal 64mer) that contained an average of $\sim$ two sulfate groups per galactose residue was synthesized and its ability to inhibit infection of cultured indicator cells by HIV-1 was compared to that of DxS (50 kDa; Fig. 4). The results show that the PS Gal 64mer inhibited HIV-1 IIIB, NL4-3, and 89.6 infectivity as well as DxS (50 kDa)—a known potent inhibitor of HIV-1 infectivity—with EC_{50} values in the nanomolar range. The cytotoxicity studies revealed that neither the glyco-dendrimer nor DxS was toxic to the cells at the highest concentration tested (3 mg/ml).

With an average of 2.3 sulfate groups per repeating glucosyl residue, it can be estimated that the 50-kDa DxS contains on average approximately 316 sulfate groups per molecule. Despite the fact that the DxS was nearly twice as large as the PS Gal 64mer and contained $\sim$ 3.7-fold-more sulfate groups, the two compounds were comparable in their ability to inhibit HIV infection of both X4 and R5 indicator cells. The fact that the galactose moieties on the dendrimer were randomly sulfated prevents the conclusion that the inhibition was due to a specific interaction between the virions and the galactose-3-sulfates on the dendrimer rather than an unspecific anionic interaction. However, even though both DxS and PS Gal 64 were randomly sulfated, both molecules had some sulfates at position C-3. Combined with the observed specificity of gp120 for SGalCer, which contains a Gal-3-sulfate moiety, one can hypothesize that positional sulfation at C-3 may be a key structural component of an inhibitor of infection by HIV-1. This hypothesis is supported by the finding that selective sulfation of 0.44 of the hydroxyls at C-3 of the 2-acetamido-2-deoxy-D-glucopyranose residues of chitin produced a compound that was a better inhibitor of infection by HIV than a compound sulfated on 0.87 of the hydroxyls at the C-6 position (35). Interestingly, sulfation at both positions 2 and 3 of the 2-acetamido-2-deoxy-D-glucopyranose residues of chitin (average of 1.7 sulfates per sugar residue) gave the best inhibitor. Therefore, even though the dendrimers were not derivatized with just galactose sulfated at C-3, it is reasonable to speculate that increasing the amount of sulfation at position C-3 of the PS Gal 64mer might result in an even more potent inhibitor of infection by HIV-1 than the one described.

The effectiveness of the PS Gal 64mer in inhibiting infection by HIV-1 coupled with its lack of cytotoxicity indicates that it merits further study for its possible use as an antagonist of HIV-1. The fact that dendrimers can be functionalized with

more than one molecule permits the development of a variety of different anti-HIV activity strategies and makes them ideal candidates for use as carriers of potential inhibitors.

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