A Convenient Oxidation of Natural Glycosphingolipids to Their “Ceramide Acids” for Neoglycoconjugation

BOVINE SERUM ALBUMIN-GLYCOSYL CERAMIDE ACID CONJUGATES AS INVESTIGATIVE PROBES FOR HIV gp120 COAT PROTEIN-GLYCOSPHINGOLIPID INTERACTIONS*

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A new method to cleave the double bond of sphingolipids has been developed. Using limited concentrations of KMnO₄ and an excess of NaIO₄, in a neutral aqueous tert-butanol solvent system gave nearly quantitative yields of the oxidized product. A variety of natural glycosphingolipids (GSLs): GlcC, GaIC, SGC, LC, Gb₂C, Gg₂C, Gb₃C, Gg₃C, Gb₅C, and GM₁C, gave the corresponding acids: 2-hydroxy-3-(N-acyl)-4-(O-glycosyl)-oxybutyric acids, i.e. “glycocol ceramide acids” (GSL, §COOH) in excellent yields (80–90%). Deacyl GSLs (dGSLs) were oxidized to acids containing the oligosaccharides devoid of hydrocarbon chains, i.e. “ceramide oligosaccharides” (dGSLNRR, COOH, where R = R₁ = H; R = H, R₁ = CH₃CO; or R = R₁ = Me). The efficacy of this method was demonstrated by transforming natural GSLs: GlcC, GaIC, GaLS, SGC, LC, Gb₂C, and Gb₃C into neoglycoproteins via coupling glycocol ceramide acids (except GaLS, which was coupled directly) to bovine serum albumin (BSA). Mass spectroscopic analysis of GaIC-BSA conjugates, (GaIC-CONH₂)BSA and (GalS-NHCO)BSA gave a value of 9 ± 1 and 16 ± 2 for n. Neoglycoconjugates derived from GlcC, GaIC (type I and II and the behenic analog), SGC, LC, and Gb₂C were recognized by the recombiant human immunodeficiency virus coat protein gp120 (rgp120). The GaIS conjugate showed significantly reduced binding, and the Gb₃C conjugate showed no binding. Thus, rgp120/GSL-BSA interaction requires a terminal galactose and/or glucose residue. Terminal N-acetylgalactosamine containing GSLs are not bound. The ceramide acid conjugates provide a more effective scaffold for presentation of glycan for rgp120 binding than those derived from dGSLs. The retention of receptor specificity of the glycoconjugates was validated by retention of the expected binding specificity of VT1 and VT₂ for Gb₃C and Gb₅C conjugates, respectively. These studies open a new vista in the generation of glycoconjugates from GSLs and further emphasize the role of aglycone in glycolipid recognition.

Glycosphingolipids form a unique amphipathic subclass of glycoconjugates present on the external leaflet of most eukaryotic plasma membranes (1, 2). A variety of functions have been ascribed to GSL,¹ including intercellular recognition (3–6), growth regulation (7, 8), differentiation (9–12), microbial adhesion (13–16), and receptors for bacterial toxins (17, 18). The sphingolipid metabolites of GSLs have also been implicated as an important new class of intracellular second messengers (19–22). In many instances, characterization of GSL function has involved purification and subsequent chemical modifications (23, 24). For example, GSL function has been investigated by coupling the free amine of dGSLs to various molecular units: fatty acids (25, 26), cross-linkers, and fluorescent probes (27–29). Additionally, the oxidative cleavage of the double bond of sphingosine has been investigated with similar objectives (24, 30–32).

The carboxylate group of the oxidized GSLs can be coupled to an amino matrix where the immobilized glycan can be used in the affinity purification of antibodies (30, 33), glycosyl hydrolases, and transferases (34). Also, the oxidized GSLs can be coupled to proteins or tailor-made polymers to yield neoglycoproteins or multivalent, high affinity glycopolymer. Although several synthetic schemes have been contrived for the generation of such multivalent glycoforms, they inevitably require the synthesis of oligosaccharide monomers containing suitable functional groups for polymerization and/or coupling (35–38). The availability of a facile method to cleavage the sphingosine double bond of a GSL or dGSL should circumvent the necessity of synthesizing such a glycan precursor. The availability of such carboxylate/amino containing derivatives provides a robust route to transform natural GSLs into various neoglycoconjugates. Synthesis of ceramide acids from natural GSLs was investigated by Hakomori and co-workers using systems such as ozone in dichloromethane (30), KMnO₄-crown ether complex in benzene (31) and KMnO₄ in acetone (32). Our attempts to use KMnO₄-crown ether-benzene system to oxidize dGSLs (GaIS or Gb₃S) gave the ceramide oligosaccharides in very low yields,

GSL; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; HPTLC, high performance thin layer chromatography; DCM, dichloromethane; t-BuOH, tert-buty alcohol; 1°ProH, iso-propyl alcohol; DCE, 1,2-dichloethane; Py, pyridine; Et₂O, diethyl ether; Bz, benzene; M, methanol; C, chloroform; A, acetone; NHS, N-hydroxysuccinimide; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; MALDI-TOF, matrix-assisted laser desorption ionization/time-of-flight; FAB, fast atom bombardment; HIV, human immunodeficiency virus; ES, Electro Spray.

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¹ The abbreviations used are: GSL, glycosphingolipid; dGSL, deacyl.

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whereas using KMnO₄ in acetonitrile gave some products (30%) with TLC migration patterns (Rᵢ values) similar to the products described in this paper. Our investigations suggested that the heterogeneity of the reaction mixture, due to solides like KMnO₄ and manganese oxide (MnO₂), affected the yield of the desired product.

Considering our particular need to oxidize dGSLs and the limited availability of natural GSLs, we required a microscale (<0.5 mm) procedure that gave the ceramide acid or ceramide oligosaccharide as the major (single) product. This study elaborates an oxidation method that fulfills these criteria.

To illustrate the biological potential of the method, we took advantage of the binding specificity of gp120 of HIV for GSLs (39–41).

**MATERIALS AND METHODS**

**Materials**

Solvents, specifically dichloromethane, tert-butyl alcohol, iso-propyl alcohol (iPrOH), 1,2-dichloro ethane, pyridine, diethyl ether, benzene, methanol, chloroform, and acetone, were purchased from either Caledon (Guelph, Ontario, Canada) or Aldrich, and ethanol (EtOH) from Commercial Alcohols Inc. (Brampton, Ontario, Canada). Reagents were purchased from the following suppliers: trifluoroacetic anhydride, K₂CO₃, sodium cyanoborohydride (NaBH₃CN), and triethylamine from Commercial Alcohols Inc. (Brampton, Ontario, Canada). Reagents from Protein Sciences Corp. (Meriden, CT), and rabbit anti-gp120 polyclonal antibody (anti-gp120-HRP) from Bglab (Burlington, MA) were purchased from Amicon®. Solvents were dried by storing over activated (silica gel, TLC, HPTLC, and aluminum-backed nanosilica plates (Alu-oxidase conjugate were from Bio-Rad. Chromatographic materials (silica gel, TLC, HPTLC, and aluminum-backed nanosilica plates (Alu-oxidase conjugate were from Bio-Rad. Chromatographic materials were purchased from Amicon®.

**Synthesis of Dimethylated Derivatives**

Gp120S-NNMe₂, Gp120S-NNMe₂ were synthesized by acetylation of Gp120 with acetic anhydride (for natural GSLs, the acetylation is performed with 1.25 mM acetic anhydride for 0.5–1 h). The peracetylated crude material was dissolved in DCE (10 ml) and loaded onto a silica column (for 3 mg, 0.5 ml for each), and the combined extracts were dried. The resulting suspension was then dissolved, by sonication, in 5 ml of distilled water. The resulting suspension was passed through a C-18 reverse phase cartridge, washed with 20 ml of water, and eluted with 20 ml of methanol. The estimated yield was >90% by TLC. Methylated compounds have reduced mobility on TLC; the Rᵢ values were 0.80 and 0.75 in (C₅H₁₂O₂, 90:35:8), respectively. Positive ion mass spectroscopy data (m/z) were as follows: GalC-NNMe₂, FAB, 489, (M+H); GalB-NNMe₂, ESI, 814 (M+H), 836 (M+Na).

**Synthesis of Trihaloacetyl Derivatives**

Gp120S-NTca, Gp120S-NTca were synthesized by acetylation of Gp120 with acetic anhydride (for natural GSLs, the acetylation is performed with 1.25 mM acetic anhydride for 0.5–1 h). The peracetylated crude material was dissolved in DCE (10 ml) and loaded onto a silica column (for each solvent composition, six 4-ml fractions were collected). The estimated yield by TLC was >90%.

**Synthesis of GalC⁺ and GalC⁻ Homologues**

To a solution of GalS (2 mg, 4 μmol) in dry pyridine (2 ml), an excess of the anhydride (approximately 5 mg, 9 or 8 μmol for oleic or erucic acid anhydrides, respectively) was added and stirred at 37 °C for 18 h. The mixture was washed with methanol (0.5 ml) and eluted with C₅H₁₂O₂ (90:35:8) and then with C₅H₁₂O₂:CH₃OH (90:10) (47). After stirring the reaction mixture for 16 h at room temperature (25 °C), methanol was removed under N₂ and the remaining solid was then dissolved, by sonication, in 5 ml of distilled water. The resulting suspension was passed through a C-18 reverse phase cartridge, washed with 20 ml of water, and eluted with 20 ml of methanol. The estimated yield was >90% by TLC. Methylated compounds have reduced mobility on TLC; the Rᵢ values were 0.80 and 0.75 in (C₅H₁₂O₂, 90:35:8), respectively. Positive ion mass spectroscopy data (m/z) were as follows: GalC-NNMe₂, FAB, 489, (M+H); GalB-NNMe₂, ESI, 814 (M+H), 836 (M+Na).

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**Synthesis of Peracetylated Derivatives**

**Method A**—Method A was suitable for natural, NaC, and NNMe₂ derivatives. A mixture of 1:2 acetic anhydride-pyridine was added to a dried sample of GSL or dGSL (final concentration of 1 mg/ml) and stirred at 37 °C for 2 h. The reactions were monitored by TLC. (H₂O, 70:30:2) showed marked enantiomer-positive products, suggesting some acylation of OH groups. Once all the Gp120S was consumed, the reaction mixture was then filtered and then was treated with water (N₂ in the solution of Et₃N·H₂O; 2.610 g/ml of GSL) and analyzed by TLC (C₅H₁₂O₂:CH₃OH:CH₃CN; 90:35:8) after stirring at room temperature for 3 h. Once all the enantiomer-positive species collapsed to a single band, the reaction mixture was stirred at 37 °C for 2 h. The reactions were monitored by TLC (DCE: isoPrOH, 80:20; 10, 3-ml fractions). The estimated yield by TLC was >90%.
was passed through a silica column (0.5 × 3 cm; C:M:H2O; 90:15:1) and analyzed in 10-mL aliquots for 4 h and the dried organic extract (see “Experimental Procedures”) was dissolved (0.5 mL, DCM:M; 2:1) and analyzed in 10-μL aliquots analyzed. In lanes 7–10, the starting material (lanes 7 and 9) and the products after premature termination at 2 h (lanes 8 and 10) are shown. Lane 1, GalS(OAc)2;NAc5-COOH; lane 2, GalS(OAc)2;NAc5-COOH; lane 3, GalS(OAc)2;NMe5-COOH; lane 4, GalS(OAc)2;NMe5-COOH; lane 5, GalS(OAc)2;NTf2-COOH; lane 6, GalS(OAc)2;Nac; lane 7, GalS(OAc)2;Nac5-COOH; lane 9, GalS(OAc)2;Nac5-COOH; lane 10, GalS(OAc)2;Nac5-COOH. Solvent system: lanes 1–6, C:M:H2O; 80:20:2; lanes 7–10, DCE:PrOH; 80:15. Comparison of lanes 7–8 and 9–10 shows that, when prematurely quenched, the reaction contains only three components: ceramide oligosaccharide (at the origin), unreacted precursor (closer to the solvent front in 8), but closer to the origin in 10), and the intermediate.

Mass Spectroscopic Analyses

The ES spectra were recorded on a Sciex API III spectrometer, FAB on a VG ZAB-SE and MALDI-TOF on a Voyager-Elite spectrometer (sinapinic acid matrix, linear mode, delayed extraction) using standard conditions.

Synthesis and Analysis of BSA Glycoconjugates

To maximize the number of glycosyl units coupled to BSA, reactions were carried out with 1.30 mol ratio of BSA to glycosyl ceramide acid. Prior to coupling, peracetylated ceramide acids were deprotected with Et3N (see oxidation protocol) and dried under N2. Residual acetate was removed by adding an acidic aqueous ethanolic solution (to 1 mg of ceramide acid was added 1 mL of 0.01 M HCl in 9:1 EtOH:H2O) and NaIO3 (30 μL, 10 μmol) and NaIO3 (15 μl, 0.75 μmol) solutions were added in the given sequence. The resulting purple mixture was stirred at room temperature and monitored by TLC every 4 h (for GSL(OAc)2, 90:15:1, C:M:H2O and for dGSL(OAc)2, 80:20:2, C:M:H2O). When clean precursors were used, catalytic regeneration of KMnO4 proceeded smoothly until the reaction was terminated. Otherwise, the purple color diminished with concomitant formation of MnO2. In such cases, additional aliquots (5 μL) of KMnO4 solution were added. The reaction was quenched by the addition of 1.5 ml of quenching solution and 1 ml of water, and the resulting colorless solution was extracted with Et2O (three times, 5 ml each). Occasionally (due to insufficient quenching) the ether extract turned yellow, and in such cases, the combined extracts were washed with 1 ml of quenching solution. The ether extract was washed with water (two times, 1 ml each) and dried under N2 at 25°C. Residual water in the crude product was removed by adding absolute EtOH (1 to 2 ml) and evaporating under N2 (Figs. 1 and 2).

For some peracetylated ceramide oligosaccharides, e.g., those with hydrophobic substitutions like Tfa or Tca (e.g., (OAc)GalSNTf2-COOH), the work-up procedure described above is applicable. However, the ceramide oligosaccharides with smaller hydrophobic substitutions such as acetyl or dimethyl (e.g., (OAc)GalSN3R3-COOH, R = Ac or Me) and charged ceramide acids ((OAc)GalS(SG3)-COOH), partitioning into Et2O was inefficient. In such cases, the reaction was quenched by adding an excess of solid NaH2SO4 (50 mg). The colorless (occasionally pale yellow) suspension was dried on a rotary evaporator and extracted (three times, 5–7 ml) with C:M:H2O; 80:20:2 and the combined extracts was passed through a silica column (0.5 × 4 cm in C:M:H2O; 80:20:2) to remove most of the salts (Fig. 2).

Deprotection of the ceramide acids or the ceramide oligosaccharides was carried out by treating dried material (0.5 mg) with triethylamine solution (1 mL of Et3N:MeOH:O2; 2:6:10) at 37°C for 2–3 h. The mixture was then dried under N2 and the residue redissolved in methanol (Figs. 3 and 4).

Ligand Binding

Neoglycoconjugates were adsorbed onto nitrocellulose membranes either directly or transferred after SDS-PAGE. The membranes were blocked with 2.5% milk powder in TBS (10 mM TBS, 100 mM NaCl) for 1 h at room temperature, rinsed three times with TBS, and incubated with rgp120 (1 μg/ml) in TBS (10 mM TBS, 50 mM NaCl) for 3 h at room temperature. The blots were washed as above, blots were incubated with the secondary antibody (1:1000) in TBS for 45 min. Finally, the blots were rinsed as above and the binding was visualized by treating with 4-chloro-1-naphthol (48) (Fig. 8).

The verotoxin binding assays were performed under conditions similar to the TLC overlay assay in which conjugates adsorbed to the nitrocellulose membrane were substituted for lipids on a TLC plate (48) (Fig. 8).
Oxidation of Glycosphingolipids for Neoglycoconjugation

RESULTS AND DISCUSSION

Oxidation Method—The chemical steps involved in the oxidation of natural GSLs is depicted in Scheme 1. This method provides the means to transform a natural GSL into a variety of neoglycoconjugates. Our objective was to develop a protocol that can be carried out with microgram quantities of GSLs, using readily available reagents. We reasoned that a procedure using "off the shelf" KMnO4 could be readily implemented, as using readily available reagents. We reasoned that a procedure that can be carried out with microgram quantities of GSLs, provides the means to transform a natural GSL into a variety of neoglycoconjugates. Our objective was to develop a protocol for catalytic regeneration of KMnO4, presumably by precipitating NaIO4.

The quantities of the reagents used in the reaction have been adjusted such that they can accommodate at least a 50% variation in the concentration of GSLs. Therefore, the procedure can be directly employed for similar quantities of different GSLs. The minimum number of equivalents of NaIO4 and KMnO4 used with respect to the GSL are 10 and 0.75, respectively, and the measured pH of the initial reaction mixture was approximately 7. Although, in principle, catalytic amounts of KMnO4 should have been adequate, using closer to 1 eq (i.e. in comparison to GSL precursor) decreased the reaction time significantly. Attempts to employ greater than 1 eq of KMnO4 resulted in the formation of brown manganese dioxide precipitates, which, in turn, led to lower yields.

Prior to the development of this new procedure, our attempts to use the previously described KMnO4-crown ether-benzene solvent system gave very low yields. For example, oxidation of 1 mg of Gb3S gave undetectable product. Further investigations suggested that the heterogeneous nature of the reaction mixture, from excess solid KMnO4 and MnO2 precipitate, was an important factor contributing to low yields. Analysis of the peracetylated GSLs showed (discussed below) that maintaining a homogeneous reaction mixture was important. We found that a tert-BuOH-water solvent system satisfied this criterion, by dissolving both the ionic KMnO4 and NaIO4 and hydrophobic peracetylated GSLs. It is important that pure peracetylated GSLs are used in the oxidation (see "Experimental Procedures"). It was observed that impurities formed during peracetylation impede the catalytic regeneration of KMnO4, presumably by precipitating NaIO4.

The steps involved in the conversion of natural GSLs into the corresponding ceramide acids and oligosaccharides. Rz = H; Rz = acyl for natural GSLs or Rz = CH3CO, CF3CO, etc., for dGSLs.

The chemical steps involved in the oxidation of Glycosphingolipids for Neoglycoconjugation
Oxidation of Glycosphingolipids for Neoglycoconjugation

Fig. 2 shows the TLCs of the protected ceramide oligosaccharides, having two reactive groups for conjugation, enables the oxidation to have selectivity toward the sphingosine double bond. The heterogeneity in the fatty acid chains. The heterogeneity in the fatty acid content of homologues gave two products in approximately 6:1 ratio (Fig. 1B). The products from erucic homologue showed higher $R_F$ value than the products from the oleic homologue. On the assumption that the product obtained from the oxidation of the sphingosine double bond, i.e. the ceramide acid, will have a greater hydrophobic character than the product from the oxidation of both double bonds (GlcC(OAc)$_6$COOH, which upon deprotection collapse to fewer bands (Fig. 3). We hypothesize that the multiple bands seen for GlcC(OAc)$_6$COOH are in part due to fatty acid heterogeneity and to the additional cleavage of double bonds present in unsaturated fatty acid chains. The heterogeneity in the fatty acid is apparently reflected in the $R_F$ to a higher degree than variation in the number of sugar residues. This hypothesis was tested by synthesizing GalC derivatives containing oleic (cis-9 octadecenoic, C18) or erucic (cis-13 docosenoic, C22) acyl chains. After oxidation each of these monounsaturated fatty acid containing homologues gave two products in approximately 6:1 ratio (Fig. 1B). The products from erucic homologue showed higher $R_F$ value than the products from the oleic homologue. On the assumption that the product obtained from the oxidation of the sphingosine double bond, i.e. the ceramide acid, will have a greater hydrophobic character than the product from the oxidation of both double bonds (GlcC(OAc)$_6$COOH, $n =$ 9 or 13), the major band is assigned to the former case. This study also suggests that the oxidation has selectivity toward the sphingosine double bond.

Glycosyl Ceramide Oligosaccharides—Ceramide oligosaccharides, having two reactive groups for conjugation, enables the synthesis of more types of glycoconjugates than ceramide acids. Fig. 2 shows the TLCs of the protected ceramide oligosaccharides derived from GalS and Gb$_3$S. The Tf$_a$ or the Tca groups can be removed to generate an amine function at an appropriate stage of glycoconjugate synthesis. Oxidation of 3GSL(OAc)$_n$ precursors proceeded at a slightly faster rate (than GSL(OAc)$_n$) and gave good yields (80%) of the oligosaccharides. The oxidation was cleaner for precursors with NAc, NTf$_a$, and NTca groups on sphingosine. The protected and deprotected oligosaccharides from dimethyl derivatives show two orcinol-positive bands (Figs. 2 and 4). We also found that treatment of GalS(OAc)$_n$-NTf$_a$COOH with Et$_3$N/M/H$_2$O for a long duration (18 h at 37 °C) gave two orcinol-positive bands with $R_F$ values comparable to ceramide oligosaccharides (Fig. 4). However, only the lower band stained with ninhydrin, consistent with the ceramide oligosaccharide GalS-NH$_2$COOH. Absence of an amine function on the second compound suggests a partial Hoffmann-type deamination (50) side reaction during the deprotection procedure.

The positive ion FAB mass spectrum of ceramide acid LC$_5$COOH is depicted in Fig. 6. Two types of ceramide acids can be identified; mass peaks at $m/z =$ 860 (M+Na, $M =$ L$_{26}$C$_{14}$COOH), 846, 832, and 818 and 804 correspond to ceramide acids having saturated fatty acids, and the peak at $m/z =$ 764 ($n =$ 18) has a hydroxylated fatty acid. Also, the spectrum shows a series of peaks resulting from the loss of a terminal sugar (loss of one glycal fragment, 162); e.g. peaks at $m/e =$ 684 arise from the molecular ion peak at 846 (M+Na) and the one at $m/e =$ 602 is from the ion at $m/z =$ 764 (M+Na).

Applications—We have shown that this oxidation method is reliable and can be used to transform small quantities of natural GSLs to novel bioactive glycosphingolipid derivatives. By way of preliminary illustration of this potential, we have coupled ceramide acids of GlcC, GalC (type I, type II, and the behenic homologue), GalS, SGC, LC, Gb$_3$, and Gb$_2$ to BSA and studied their interaction with HIV coat protein rgp120 (40, 51, 52) and veroxtoxins (17, 53). The pathway of HIV entry into T-lymphocytes is initiated by the binding of gp120 to the cellular CD4 receptor. This induces a conformational change in gp120 and causes a second interaction with a chemokine receptor. These events eventually lead to the fusion of viral and cellular membranes (54, 55). However, GSLs, specifically GalC and SGC, have been implicated in the HIV infection of CD4-negative tissues such as fibroblasts and neural and intestinal epithelial cells (40, 51, 56, 57). A class of synthetic GalC mimics inhibited the interaction of rgp120 to suramin and inhibited the infection of HIV-1 in human peripheral blood mononuclear cells (58). We used veroxtox-GSL interactions as a means to verify the specificity of our glycoconjugates (discussed later) (17, 53).

Deprotected ceramide acids were coupled to BSA using the carbodiimide-NHS system. A molar ratio of 30:1 of the ceramide acid to BSA, they appear as diffused bands. Also, the gels show two sets of bands, presumably corresponding to monomeric (≈ 80 kDa) and dimeric conjugates (close to the gel front).

The number of galactosyl ceramide acid units per BSA was estimated using MALDI-TOF mass spectroscopic analysis (Fig. 6). Conjugates (GalC$_5$CO), BSA and (GalS-NH)$_2$BSA gave broad peaks around 82 and 83 kDa, respectively. However, BSA treated with EDAC-NHS alone gave a peak at 75 kDa, (compared with 66 kDa for BSA), probably due to the coupling of small peptide impurities present in BSA. Therefore, 75 kDa was considered as the mass for uncoupled BSA. Shifts of 6.1 and 7.2 kDa for (GalC$_5$CO)$_2$BSA and (GalS-NH)$_2$BSA conjugates correspond to a mean value of 9 and 16 respectively. On the assumption that the broader peaks of the conjugates (as compared with the uncoupled BSA) result from variation in $n$, the value of $n$ was calculated to be $n =$ 16 + 2 and $n =$ 9 + 1 for (GalS-NH)$_2$BSA and (GalC$_5$CO)$_2$BSA, respectively. In the case of (GalC$_5$CO)$_2$BSA, a (weighted) average molecular weight was used for the ceramide acid GalC$_5$COOH. The value of $n$ obtained by this analysis for (GalS-NH)$_2$BSA is consistent with a recent report, where 17 globo H antigens (monomers with an aldehydic spacer) were coupled to BSA by reductive amination (59). Investigating various coupling conditions showed that the amine function of dGSLs couples readily, whereas the coupling
of ceramide acids is more difficult (data not shown).

A Western blot analysis, where the glycoconjugates were first separated by SDS-PAGE, transferred to nitrocellulose membrane and probed for rgp120 binding is shown in Fig. 7. Due to the diffuse nature of the monomeric conjugates, the binding profile of rgp120 is also diffuse, whereas the high molecular weight conjugates show stronger signals. Qualitatively, GalC, SGC, and LC conjugates bind rgp120, whereas GalS showed reduced binding, and Gb4C no binding. In the case of GalC\textsubscript{2}CCOOH and GalS conjugates, since the substitution per BSA is known, a quantitative interpretation of binding can be made. Although nearly twice as many GalS units were coupled per BSA than GalC\textsubscript{2}CCOOH, the ceramide acid conjugate is a better ligand for rgp120.

Dose responses for the binding of BSA conjugates to rgp120 by dot blot are shown in Fig. 8. Conjugates derived from GlcC, GalC, Ga10\textsuperscript{C}C, Gal\textsuperscript{C}, SGC, LC, and Gb\textsubscript{3}C showed dose-dependent binding, whereas Gb\textsubscript{4}C did not bind. The binding profiles of the Ga1S conjugate consistently showed less binding than the ceramide acid conjugates. These findings differ significantly from the binding observed for intact GSLs adsorbed on TLC plates in which, consistent with the literature (39, 40, 56, 57), only GalC and SGC bound rgp120. We propose that the presentation of carbohydrate is different in these two cases (i.e., the natural GSL on TLC versus neoglycoconjugate), modulated by the aglycone.

Some natural GSLs, such as bovine GalC, have a significant fraction of unsaturated fatty acids, which give rise to dicarboxylic acids after oxidation. For such species, coupling could occur at the distal acid group of the acyl chain or at the ceramide acid. To ensure that the coupled ceramide acid is recognized by rgp120, a GalC homologue containing only behenic acid was synthesized and transformed into the corresponding conjugate. The behenic conjugate showed a binding profile comparable to the conjugates derived from natural type I and type II GalC.

To authenticate the Gb\textsubscript{3}C and Gb\textsubscript{4}C conjugates, a dose-response dot blot binding analysis (Fig. 8) of Gb\textsubscript{3}C, Gb\textsubscript{4}C and GlcC BSA conjugates with verotoxins VT1 and VT2\textsubscript{a} was performed. It is well established that VT1 is highly specific for Gb\textsubscript{3}C, whereas VT2\textsubscript{a} binds to both Gb\textsubscript{3}C and Gb\textsubscript{4}C (60). Neither toxin binds to GlcC. The blots clearly showed that these conjugates bind to VTs with the expected selectivity, and their binding profiles are consistent with multiple binding sites of the toxin interacting with a multidentate ligand.

Three conclusions can be made from these studies involving rgp120. First, the binding specificity for the neoglycoconjugate is distinct from that for the free GSL. Neither GlcC or Gb\textsubscript{3}C are bound, but their BSA conjugates are efficiently recognized. The binding of Gb\textsubscript{3}-BSA is of particular interest in light of the role of Gb\textsubscript{3} in HIV-induced cell fusion (61), suggesting that the “molecular environment” may modulate Gb\textsubscript{3} binding. Second, glycoconjugates derived from ceramide acids of GalC are more effective receptors than those derived from the dGSL, GalS, indicating the utility of this oxidation procedure. This could be exploited in the design of soluble GSL mimetics. For example, ceramide acids having optimized acyl units could be coupled to dendrimers or to defined peptides. Third, our studies show that when GSLs are transformed into a neoglycoprotein scaffold, rgp120 recognizes epitopes with terminal galactose or glucose.
but does not bind ligands containing terminal N-acetylgalactosamine. This is consistent with the observed binding profile of the variable loop-derived (V3) synthetic peptide SPC3, which showed very low affinity toward terminal GalNAc containing Gb4 or GM2 but bound to SGC, LC, GM3, and GD3 (51).

Our finding that GlcC-BSA is a good gp120 receptor is not explained by current concepts that govern the specificity of protein/carbohydrate interaction. The binding of both GalC and GlcC conjugates with rgp120 suggests that, in this context, the 4-OH of these hexoses may not play a direct role in binding. If we assume that rgp120 is a monomer and has only one carbohydrate binding domain, a possible explanation for the altered binding specificity might be carbohydrate-carbohydrate interaction within the GalC or GlcC conjugates. Since Gb3 and LC BSAs are bound, it may be that the binding domain of rgp120 recognizes a linear disaccharide unit (i.e. 1–4 type arrangement) containing a Gal-Gal, Glc-Glc, or Gal-Glc sequence. In the case of Gb3C and LC conjugates, such arrangement is covalently established within each oligosaccharide unit. In GalC and GlcC conjugates, hydrogen bonding between the glucosidic oxygen of one sugar and the 4-OH (irrespective of whether axial or equatorial) of the second sugar could give a similar hexose-hexose arrangement, particularly along the edges of the pyranose rings (rather than the planes of the two rings). We observed that the rgp120 binding to GSL conjugates, particularly for the monohexose conjugates, was significantly better at lower salt concentration (50 mM versus 100 mM). Higher salt concentrations might disrupt such carbohydrate-carbohydrate interactions mediated by direct hydrogen bonding.

In summary, we have developed a new procedure for the microscale oxidation of the sphingosine double bond, which gives a high yield of the desired carboxylic acid. This procedure will allow a new systematic approach to the generation of GSL mimics that can be used in the investigation of GSL-protein interactions and design of possible therapeutic agents.

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FIG. 8. Dose response for rgp120 and verotoxin BSA neoglycoconjugate binding. The highest concentration for each conjugate is 2 μg of protein (corresponding to less than 200 ng of glycosylceramide acid). All conjugates are numbered according to Fig. 7. Upper panel, dot blot binding of glycoconjugates (0.5 dilutions) and rgp120, where the bound rgp120 was detected by human or rabbit antisera. The rgp120 binding of conjugates 2–5 is quantitated in the inset by pixel integration. Data in solid lines are for human antiserum and, in the dotted lines, for rabbit antiserum. Open symbols, GalS conjugates; solid symbols, GalC-COOH conjugates. Lower panel, dose-response analysis involving glycoconjugates (0.2 dilutions) and verotoxins. Controls for goat anti-rabbit (GAR) and goat anti-human (GAH) serum alone showed no binding.
