The P Histo-Blood Group-Related Glycosphingolipid Sialosyl Galactosyl Globoside as a Preferred Binding Receptor for Uropathogenic *Escherichia coli*: Isolation and Structural Characterization from Human Kidney†

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ABSTRACT: The P histo-blood group-related glycosphingolipid, sialosyl galactosyl globoside (SGG), has recently been implicated as a preferred binding receptor for uropathogenic *Escherichia coli* [Stapleton, A. E., Stroud, M. R., Hakomori, S., and Stamm, W. E. (1998) *Infect. Immun. 66,* 3856−3861]. We report here the purification and complete structural characterization of SGG from normal human kidney. Using metabolically [^{35}S]-labeled *E. coli* as a probe, a monosialylated glycosphingolipid was isolated to homogeneity. The glycosphingolipid was purified by a combination of high-performance liquid chromatography and preparative high-performance thin-layer chromatography and its structure unambiguously elucidated by ^{1}H NMR, electrospray ionization mass spectrometry, and methylation analysis. Its primary structure was shown to be identical to a previously characterized, developmentally regulated, globo-series glycolipid thought to be unique to human teratocarcinoma. The significance of this structure as a unique receptor in human kidney for uropathogenic *E. coli* and its role in the pathogenesis of urinary tract infections are discussed.

Nearly all of the antigenic determinants defining the P histo-blood group system (P^k, P, LKE, and P_1) are associated with globo-series glycosphingolipids (*1*, *2*). Globo-series glycosphingolipids (GSLs) are defined at the chemical level as having the trisaccharide Galα1→4Galβ1→4Glcβ1 (P^k) attached at the reducing end to ceramide (*N*-fatty acyl sphingosine) (Table 1). This "globo-core" can be further glycosylated to form more complex carbohydrate structures that define additional P blood group related antigens, i.e., P and LKE (*3*), as well as those antigens associated with the ABO histo-blood group system (*4*). P blood group antigenic structures are strictly carried on glycosphingolipids (*5*) and play an important role in the pathogenesis of urinary tract infections, particularly as receptors for uropathogenic *Escherichia coli* (*6*). The Galα1→4Gal sequence found in all blood group P antigens is the minimal structure required for binding P fimbria-expressing uropathogenic *E. coli* (*7*, *8*). However, women expressing P blood group related antigens who carry ABH blood group antigens in secretions (secretors) have a lower incidence of urinary tract infections (UTI)[1] than individuals (nonsecretors) who do not express these determinants (*9*−*11*). It has been hypothesized that the increased risk of UTI in nonsecretors and the increased attachment of uropathogenic bacteria to their uroepithelial cells (*11*) is due to the expression of a unique receptor for *E. coli* (*12*). Evidence suggests that nonsecretors selectively express two extended globo-series GSLs, sialosyl galactosyl globoside (SGG) and disialosyl galactosyl globoside (DSGG), which specifically bind uropathogenic *E. coli* (*12*), and that one of these GSLs (SGG) is the preferred receptor over other globo-series GSLs for *E. coli* isolates expressing a P-related adhesin (*13*).

Since globo-series glycolipids have been shown to be modified by histo-blood group status, and the globo-core is the major carrier isotype (type 4 chain) of ABH active epitopes in renal epithelium (*46*), we hypothesized that the increased risk of UTI in nonsecretors may be due to the presence of a unique receptor for P-fimbriated *E. coli* in these patients. In the present study, using a metabolically [^{35}S]-labeled clinical *E. coli* isolate (R45) as a probe, a monosialylated ganglioside comigrating with a sialosyl galactosyl globoside standard by high-performance thin-layer chromatography (HPTLC) in two different solvent systems was purified from normal human kidney. Its structure was

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[1] Abbreviations: Cer, ceramide; C/M, chloroform/methanol; Fuc, L-fucose; Gal, D-galactose; Glc, D-glucose; GalNAc, *N*-acetyl-D-galactosamine; GlcNAc, *N*-acetyl-D-glucosamine; ^{1}H NMR, proton nuclear magnetic resonance; HPTLC, high-performance thin-layer chromatography; HPLC, high-performance liquid chromatography; IHW, isopropyl alcohol−hexane−water; MAb, monoclonal antibody; NeuAc, *N*-acetylneuraminic acid; PBS, phosphate buffered saline; SGG, sialosyl galactosyl globoside; SSEA-4, stage specific embryonic antigen 4; UTI, urinary tract infection. Glycolipids are abbreviated according to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [*Lipids* (1977) *12,* 455−463]; however the suffix −OseCer is omitted.

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Table 1: Structures of Glycosphingolipids Referred to in This Study[a]

glycosphingolipid	structure	P blood group activity
CTH	Galα1−4Galβ1−4Glcβ1−1Cer	P^k
Globoside	GalNAcβ1−3Galα1−4Galβ1−4Glcβ1−1Cer	P
Gal-globoside	Galβ1−3GalNAcβ1−3Galα1−4Galβ1−4Glcβ1−1Cer	
Forssman	GalNAcα1−3GalNAcβ1−3Galα1−4Galβ1−4Glcβ1−1Cer	
Globo-H	Fucα1−2Galβ1−3GalNAcβ1−3Galα1−4Galβ1−4Glcβ1−1Cer	
Globo-A	GalNAcα1−3(Fucα1−2)Galβ1−3GalNAcβ1−3Galα1−4Galβ1−4Glcβ1−1Cer	
P^1	Galα1−4Galβ1−4GlcNAcβ1−3Galβ1−4Glcβ1−1Cer	P_1
SGG	NeuAcα2−3Galβ1−3GalNAcβ1−3Galα1−4Galβ1−4Glcβ1−1Cer	LKE
DSGG	NeuAcα2−3Galβ1−3(NeuAcα2−6)GalNAcβ1−3Galα1−4Galβ1−4Glcβ1−1Cer	

[a] Key: CTH, ceramide trihexoside (Gb_3, globotriaosylceramide); globoside, Gb_4 (globotetraosylceramide); gal-globoside, galactosyl globoside, Gb_5, SSEA3; SGG, sialosyl galactosyl globoside, SSEA4; DSGG, disialosyl galactosyl globoside; Cer, ceramide; Fuc, L-fucose; Gal, D-galactose; Glc, D-glucose; GalNAc, *N*-acetyl-D-galactosamine; GlcNAc, *N*-acetyl-D-glucosamine; NeuAc, *N*-acetylneuraminic acid.

unambiguously confirmed by ^{1}H NMR, electrospray ionization mass spectrometry (ESI-MS), and methylation analysis as V^3NeuAcGb_5Cer (SGG), a developmentally regulated globo-series glycosphingolipid previously thought to be unique to human teratocarcinoma and sharing the same terminal epitope as the stage-specific embryonic antigen, SSEA-4. To our knowledge, this is the first report describing the complete and unambiguous structural characterization of SGG from normal human tissue. Its identification in human kidney, a target organ for uropathogenic *E. coli* infection, and its role as a high affinity ligand (*13*) may explain the chemical basis for the increased risk of UTI in nonsecretors.

MATERIALS AND METHODS

Preparation of Human Kidney Ganglioside Fraction. (A) Glycolipid Extraction. Approximately 800 g of pooled human kidney tissue was extracted by homogenization (*14*) in a Waring blender with 10 volumes of the lower phase of 2-propanol:hexane:water (IHW; 55:25:20). The extract was filtered through a Whatmann No. 1 filter and the residue reextracted and filtered as above. The extraction/filtration procedure was repeated once more, and the combined filtrates were concentrated under reduced pressure at 40 °C using a Brinkman rotary evaporator. The concentrated extract was subjected to Folch partitioning by dissolving the residue in 3 L of C/M (2:1) containing 500 mL of water. After vigorous shaking, the extract was allowed to separate until two translucent phases appeared (*15*). The upper phase was removed and the lower phase reextracted by the addition of C/M/1% KCl (1:10:10) to the original level. The liquid−liquid extraction procedure was repeated two times, and the combined upper phases were concentrated by rotary evaporation, reconstituted in water, and dialyzed exhaustively against deionized water using Spectropor 3 dialysis tubing (MW cutoff = 3500).

(B) Anion Exchange Chromatography. After dialysis, the upper-phase extract was evaporated to dryness as above and dissolved in 50 mL of C/M/water (30:60:8) by a combination of warming at 37 °C and sonication. Insoluble material was removed by centrifugation at 1000g for 10 min and reextracted by sonication in an additional 50 mL of the same solvent. Following centrifugation as above, the combined supernatants were loaded onto a DEAE-sephadex column (300 mL bed volume; acetate form) and washed with 2 L of C/M/water (30:60:8) to remove all neutral lipids (*16*). The column was equilibrated with 500 mL of methanol and the ganglioside fraction eluted with 2 L of 1.0 M NH_4OAc in methanol. The eluted ganglioside fraction was dried by rotary evaporation, dialyzed against water, and dried as above.

Purification of Sialosyl Galactosyl Globoside from Human Kidney Ganglioside Fraction. (A) High-Performance Liquid Chromatography. The ganglioside fraction was solubilized in 10 mL of IHW and transferred from the evaporation flask to a 15 mL tube. The sample was completely dried under N_2 at 37 °C using a nitrogen evaporator (N-EVAP, Organomation Inc., South Berlin, MA) and reconstituted in 2 mL of IHW by sonication. The sample was injected onto a preparative Iatrobead column (6RS-8010; 0.8 × 60 cm; Iatron Laboratories Inc., Kanda/Tokyo, Japan) preequilibrated with IHW (55:40:5), and subjected to a linear gradient from IHW 55:40:5 to 55:25:20 with a flow rate of 1 mL/min (*17*). Fractions of 4 mL each were collected over 400 min. Each fraction was spotted onto an HPTLC plate, developed in chloroform/methanol/0.5% $CaCl_2$ (described below), and visualized by spraying with 0.5% orcinol in 2 N sulfuric acid. A parallel plate was developed and used in bacterial overlay assays (see below). Fractions staining positive to orcinol and *E. coli* were pooled according to migration. HPLC fractions 39−48, showing the strongest binding by the *E. coli* overlay assay and containing more than one band by TLC, were pooled, dried under N_2, resolubilized in 1 mL of IHW, and injected onto a semipreparative Iatrobead column (0.4 × 60 cm). A linear gradient from IHW 55:40:5 to 55:25:20 over 200 min with a flow rate of 0.5 mL/min was used. Fractions of 1 mL each were collected, assayed, and pooled as described above. One pool (fractions 53−58), showing the strongest staining by *E. coli* and containing multiple bands by TLC, was further purified by preparative HPTLC (described below).

(B) High-Performance TLC. The strongest staining band in fraction 53−58 was separated by preparative HPTLC in chloroform/methanol/0.5% $CaCl_2$ (50:40:10) so only a single orcinol and *E. coli* positive band was observed. The sample was further resolved into three additional bands by HPTLC using a solvent system of 1-propanol/water/NH_4OH (6:1.5:1). Preparative TLC was performed by streaking 50 μL of sample across a 10 × 20 cm HPTLC silica gel plate (silica gel 60; EM Science, Gibbstown, NJ), drying, and developing in the appropriate solvent system. Plates were dried, and bands were visualized by spraying with 0.03% primulin in 80% acetone. Bands were marked with a pencil under UV light. Marked bands were scraped from the plate using a razor blade, and the gangliosides were extracted from the silica

by sonicating for 20 min in IHW (55:25:20; 2 mL per band). The silica was removed by centrifuging at 1000g for 10 min and reextracted as above, and the combined supernatants were dried under N_2. Samples were cleaned up using 1 cm^3 tC-18 Sep-Pak cartridges (Waters, Milford, MA) by first dissolving the sample in 1 mL of PBS and then applying it to a column equilibrated with PBS after sequentially washing with 5 mL of methanol and 5 mL of water. Once the sample was retained, the column was washed with 10 mL of water, followed by 10 mL of 50% methanol, and eluted in 5 mL of 100% methanol. The sample was dried under N_2, dissolved in 1 mL of IHW (55:25:20), and injected onto an Iatrobead column (0.4 × 30 cm) as above using a linear gradient from IHW 55:40:5 to 55:25:20 for 100 min at a flow rate of 1 mL/min. Fractions of 1 mL each were collected and visualized by HPTLC using the orcinol−sulfuric acid reaction. Orcinol−positive fractions were pooled and dried under N_2 prior to structural analysis.

Bacterial Overlay Assays. Assays were performed as previously described (*12*) using metabolically [^{35}S]-labeled *E. coli* isolate R45, a wild-type cystitis isolate (*18*), which expresses P fimbriae carrying the class II *pap*-encoded adhesin (*19*) and binds globo-series glycolipids.

^{1}H-Nuclear Magnetic Resonance Spectroscopy. A sample of the ganglioside was prepared for NMR analysis by repeated lyophilization from D_2O (99.996 at. %; Cambridge Isotope Laboratories, Woburn, MA) and then dissolved in 0.5 mL DMSO-d_6 (99.96 atom %; Aldrich, Milwaukee, WI) containing 2% D_2O. All ^{1}H NMR spectra were acquired at 600 MHz on a Bruker (Karlsruhe, Germany) DRX-600 Fourier transform spectrometer, at a probe temperature of 308 K and a sweep width of 3600 Hz and with suppression of the residual HOD resonance by a presaturation pulse during the preparatory delay period. One-dimensional ^{1}H NMR spectra were resolution enhanced by exponential multiplication (LB = 0.3) prior to Fourier transformation. Acquisition and processing of 2-D TPPI−COSY, −TOCSY, and −NOESY experiments were performed essentially as described previously (*20*, and references cited therein).

Positive Ion Mode Electrospray Ionization Mass Spectrometry. ESI-MS experiments were carried out on a PE-Sciex API−III spectrometer with IonSpray source. A sample of ganglioside was permethylated as described previously (*21*), dissolved in methanol containing 1 mM ammonium acetate, and introduced by direct infusion. For single quadrupole spectra, the mass range *m*/*z* 100−2200 was scanned at an orifice-to-skimmer potential of 180 V, or, for a higher resolution spectrum of the pseudomolecular ion region, *m*/*z* 2000−2250 at 200 V. For tandem ESI-MS/CID-MS experiments, the orifice-to-skimmer potential was lowered to 120 V to increase the abundance of disodiated, doubly charged pseudomolecular ions; argon was introduced into the collision cell at CGT ≅ 400, and precursor ions were selected in Q_1, while the mass range *m*/*z* 100−2200 was scanned in Q_3.

Linkage Analysis by GC-MS. An aliquot of permethylated ganglioside was depolymerized, reduced, and acetylated essentially as described (*21*). Analysis of the resultant partially methylated alditol acetates (PMAAs) was performed on a Hewlett-Packard 5890 GC/5970 MSD operating in the splitless mode, using a 30 m DB-5 bonded phase fused silica capillary column, temperature programmed from 160 to 260 °C at 2 °C/min. PMAA derivatives were identified by retention times and characteristic EI mass spectra (*22*, *23*) compared with those of authentic standards.

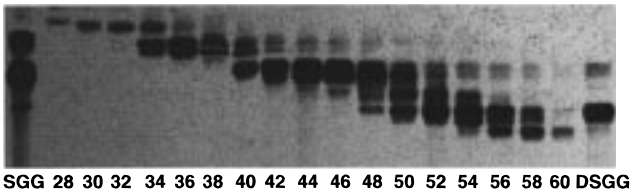


FIGURE 1: HPTLC of ganglioside fractions of normal human kidney separated by HPLC in CMW solvent system 50:40:10. Total ganglioside fraction from human kidney was separated by normal phase preparative HPLC using a linear gradient from IHW 55:40:5 to 55:25:20. Fractions were collected and separated by HPTLC in CMW (50:40:10). Bands were revealed by overlaying HPTLC plate with metabolically [^{35}S]methionine-labeled *E. coli* isolate R45. SGG, sialosyl galactosyl globoside standard; DSGG, disialosyl galactosyl globoside standard. Fractions 39−48, comigrating with SGG standard was pooled and subjected to a second HPLC (semipreparative HPLC, see text). Autoradiograph is shown.

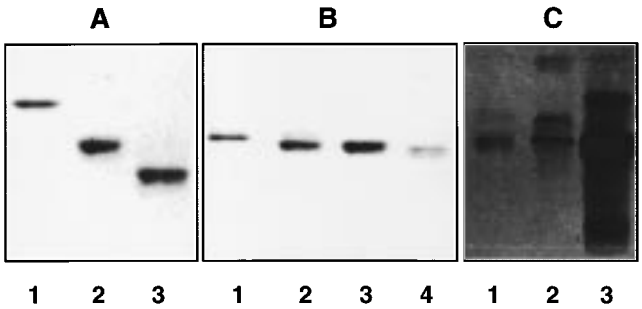


FIGURE 2: HPTLC of purified gangliosides from human kidney. HPTLC plates of three unknown gangliosides purified from pooled HPLC fraction 53−58 (from semipreparative HPLC). Panel A, pooled ganglioside fraction (53−58) previously migrating as a single band in CMW system is resolved into three bands using a solvent system of 1-propanol/water/NH_4OH (6:1.5:1) after preparative TLC in the same solvent system; stained with orcinol/sulfuric acid reagent. Panel B, the same three gangliosides shown in panel A developed in CMW (50:40:10) and stained with orcinol/sulfuric acid reagent. Panel C, autoradiograph of HPTLC plate identical to the samples and solvent conditions used in panel B stained with [^{35}S]-labeled *E. coli* isolate R45. Ganglioside fraction 3 (lane 3) showing strong staining with *E. coli* was structurally characterized.

RESULTS

Purification of SGG from Normal Human Kidney. The HPLC elution pattern of *E. coli*-binding gangliosides as determined by HPTLC is shown in Figure 1. Fractions 39−48 were pooled on the basis of strong binding to [^{35}S]-labeled *E. coli* and comigration with an SGG standard prepared from partially desialylated DSGG. Pooled fraction 39−48 was subjected to a second HPLC as described in Materials and Methods. Fractions 53−58 obtained after the second HPLC were pooled on the basis of the same criteria described above. A single band comigrating with the SGG standard in C/M/0.5%$CaCl_2$ (50:40:10) was obtained after preparative HPTLC in the same solvent system (data not shown). The GSL sample was further resolved into three components and purified by preparative TLC in a solvent system consisting of 1-propanol/water/NH_4OH (6:1.5:1). A thin-layer chromatogram showing the separation of the three GSL components after preparative TLC in the above solvent system is shown in Figure 2A. The three GSL fractions were labeled according to their migration in 1-propanol/water/NH_4OH (6:

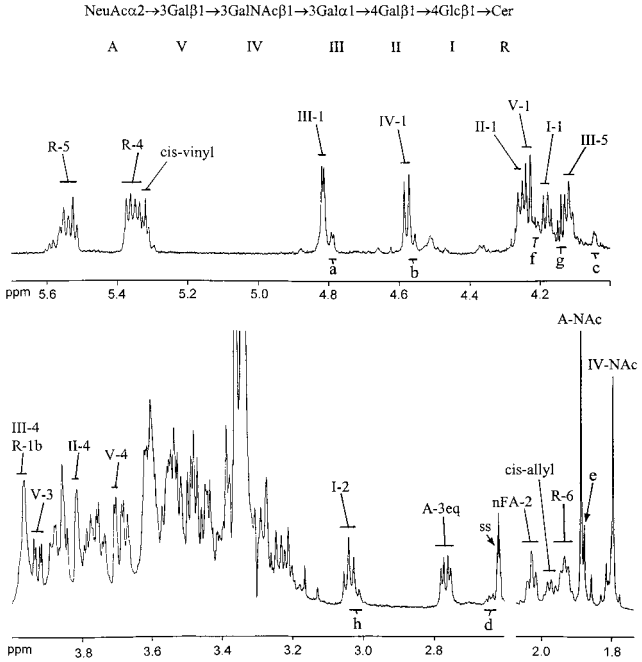


FIGURE 3: Selected regions of a 1-D proton NMR spectrum of the monosialosyl ganglioside from human kidney in DMSO-d_6/D_2O at 308 K. Region from 1.7 to 2.1 ppm is attenuated ×0.4. Arabic numerals refer to ring protons of residues designated by Roman numerals or capital letters in the corresponding structure drawn at the top of the figure. R refers to protons of the sphingosine backbone only; cis-vinyl and cis-allyl refer to protons of unsaturated *N*-fatty acyl chains; nFA-2 refers to H-2 of non-hydroxylated *N*-fatty acyl chains. Resonances from minor components are designated by small letters and assigned as follows: a−e, III-1, IV-1, III-4, A-3eq, and B-NAc, respectively, of a monosialoganglioside component proposed to have an isomeric structure with NeuAc (B) linked α2→6 to *β*-GalNAc IV of galactosylgloboside; f and h, I-1 and I-2 of ganglioside component with 2-hydroxy fatty acyl chains; g, unknown impurity.

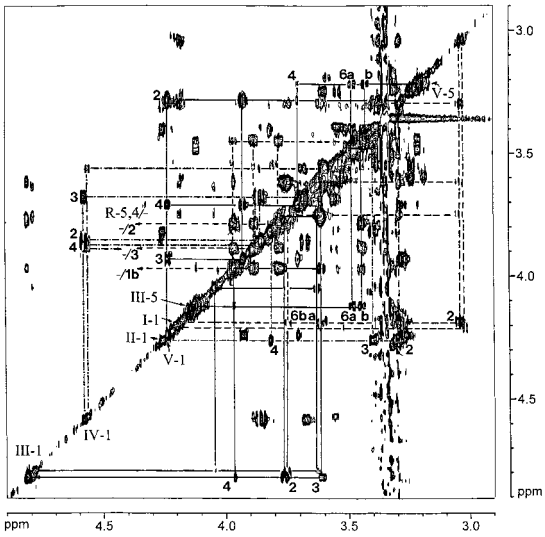


FIGURE 4: Downfield region of 2-D TOCSY spectrum of the monosialosyl ganglioside from human kidney in DMSO-d_6/D_2O at 308 K. Monosaccharide residue H-1 and selected H-5 resonances are designated on diagonal by Roman and Arabic numerals as in Figure 3. Off-diagonal correlations are marked by Arabic numerals only. Upper left section: connectivities originating from V-1 and V-5 (solid lines), R-5 and R-4 (dashed lines), and IV-1 (dashed/dotted lines). Lower right section: connectivities originating from III-1 and III-5 (solid lines), I-1 (dashed lines), and II-1 (dashed/dotted lines). Connectivities originating from minor components are shown for III-1, IV-1, and I-1, resonances a, b, and f, respectively in Figure 3.

1.5:1); i.e., the fastest migrating component was labeled 1 and the slowest 3. All three samples were rechromatographed in C/M/0.5%$CaCl_2$ (50:40:10) on duplicate plates. Figure 2B shows a thin-layer chromatogram stained for carbohydrates by the orcinol−sulfuric acid reaction. Figure 2C shows the same chromatogram as in Figure 2B with the bands revealed by [^{35}S]-labeled *E. coli*. Fraction 3, which comigrated with the SGG standard (Figure 2B) and showed the strongest binding by *E. coli* (Figure 2C), was subjected to structural analysis.

^{1}H-NMR Spectroscopy. Portions of the 1-D ^{1}H NMR spectrum of the monosialosyl ganglioside from human kidney are reproduced in Figure 3. Although a number of signals from glycosphingolipid and other impurities can be observed, the spectrum of the major component is clearly similar to that obtained previously from V^{3}NeuAcGb$_5$Cer (GL-7)

Table 2: Proton Chemical Shifts (ppm from Tetramethylsilane) and $^3J_{1,2}$ Coupling Constants (Hz) for Sialosylgalactosylgloboside in Dimethyl Sulfoxide-d_6/2% D_2O at 308 K

	NeuAcα2⟶3Galβ1⟶3GalNAcβ1⟶3Galα1⟶4Galβ1⟶4Glcβ1⟶1Cer						
	A	V	IV	III	II	I	R
H-1		4.234	4.579	4.815	4.256	4.185	3.447 (a)
($^3J_{1,2}$)		(7.9)	(8.3)	(4.2)	(7.5)	(7.7)	3.962 (b)
H-2		3.279	3.858	3.766	3.310	3.040	3.781
H-3	2.766 (eq) 1.346 (ax)	3.926	3.675	3.609	3.399		3.880
H-4	3.538	3.706	3.880	3.964	3.815		5.354
H-5				4.117		3.290	5.538
H-6				3.476 (a) 3.454 (b)		3.611 (a) 3.747 (b)	1.935
Nac	1.889		1.795				

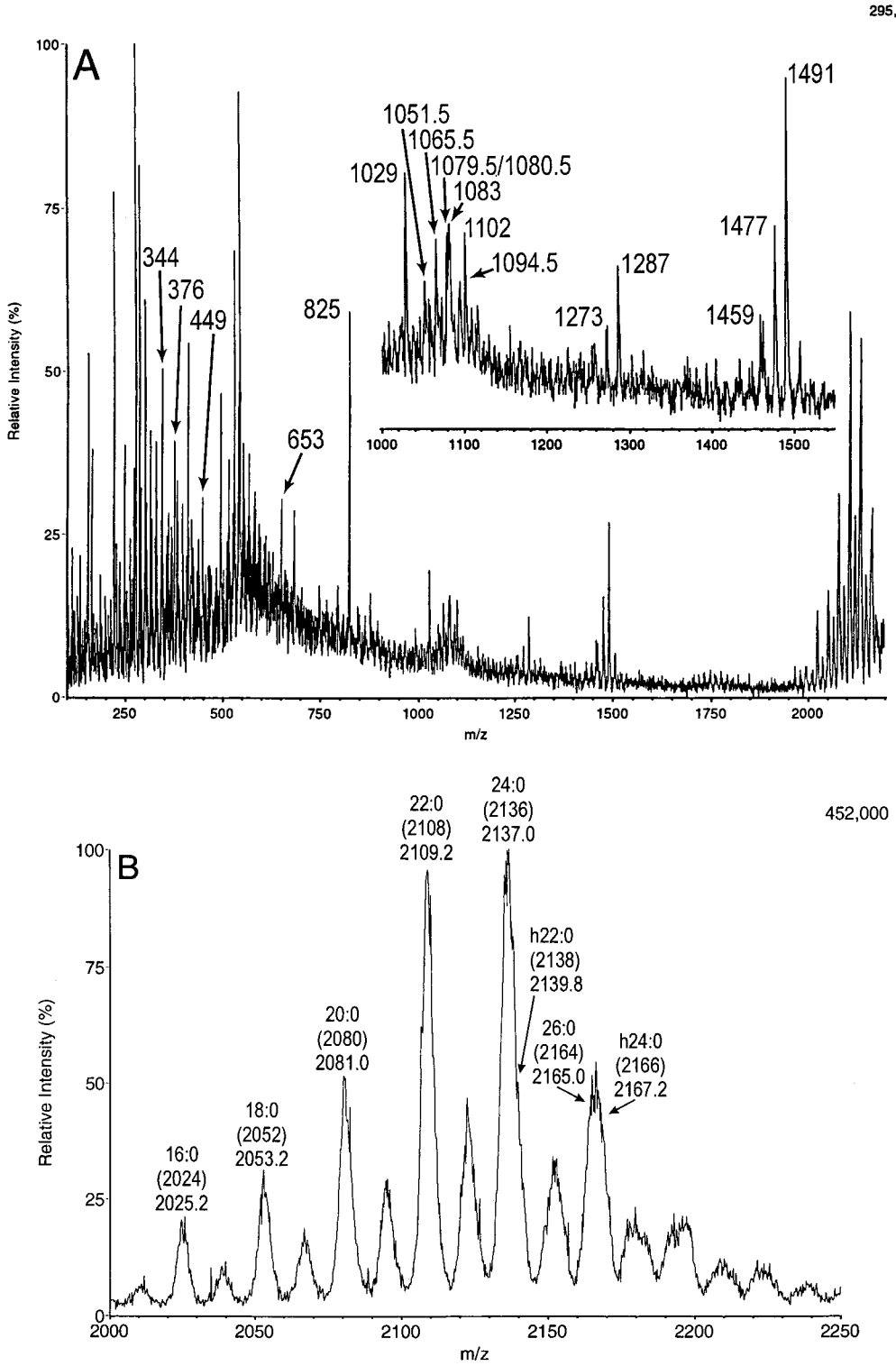


FIGURE 5: Positive ion mode ESI-CID-MS (orifice-to-skimmer potential = 180 V) of permethylated monosialosyl ganglioside from human kidney. Panel A, mass range *m*/*z* 100−2200. Inset, expansion of *m*/*z* 1000−1550; relevant fragments are designated by nominal monoisotopic mass (see Scheme 1). Panel B, mass range *m*/*z* 2000−2250 scanned at higher resolution; monosodiated, singly charged, pseudomolecular ions are designated by fatty acid species (top), nominal monoisotopic mass (parentheses), and measured *m*/*z* (to nearest 0.1 u).

originally isolated from the human teratocarcinoma cell line 2102Ep (*24*). With respect to anomeric and other structural reporter resonances, the chemical shift differences are all <0.01 ppm. The sugar residues of the core glycan are represented by five anomeric resonances, four β ($^3J_{1,2} = 7-9$ Hz) and one α ($^3J_{1,2} = 2-4$ Hz), as expected for a Gb_5 pentasaccharide. The chemical shifts of two β-anomeric signals (4.185 and 4.256 ppm) and the single α-anomeric signal (4.815 ppm) are very close to those of β-Glc I, β-Gal II, and α-Gal III, respectively, in Gb_4Cer and Gb_5Cer measured under similar conditions (*24*, *25*). The remaining β-anomeric resonances (4.579 and 4.234 ppm) correspond to those for β-GalNAc IV and β-Gal V, respectively, in Gb_5Cer, with a relative upfield shift for the former (−0.03 ppm) and a downfield shift for the latter (+0.04 ppm). These are the glycosylation-induced shift changes expected for

addition of NeuAcα2→3 to a terminal Galβ1→3/4HexNAc group (*26*, *27*). The additional NeuAc residue is distinguished by an H-3eq signal (2.766 ppm, dd) and an NAc signal (1.889 ppm, s, 3H) at chemical shifts diagnostic for an α2→3 linkage to terminal β-Gal (*26*, *27*). A second NAc signal (1.795 ppm, s, 3H) could be assigned to the β-GalNAc residue; it was observed previously at 1.797 ppm in the spectrum of V^3NeuAcGb$_5$Cer (*28*).

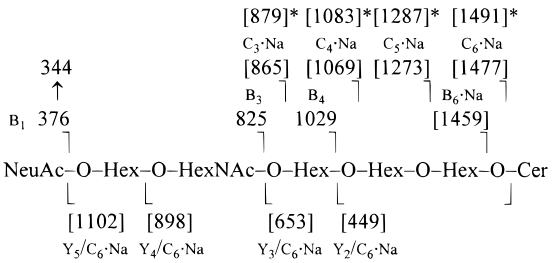
To substantiate the proposed glycan structure, as well as confirm the proton resonance assignments, the sample was subjected to a series of 2-D NMR experiments, including PS-DQF-COSY, TOCSY (see Figure 4), and NOESY. This allowed assignment of H-1 through H-4 of all three β-*galacto*pyranoside spin systems, H-1 through H-6 of both the α-*galacto*pyranoside and the β-*gluco*pyranoside spin systems, and H-3 through H-5 of the NeuAc spin system, along with the functionalized proximal part of the ceramide spin system. Because of the vanishingly small $^3J_{4,5}$ coupling in galactopyranosides, which attenuates transmission of coherence between H-4 and H-5 of a *galacto*pyranoside (*29*−*31*), it was not practical to assign H-5 and H-6 resonances for these spin systems, except in the case of α-Gal III, where the distinct downfield position of H-5 allows a connectivity to be established with the remaining ring protons in the TOCSY experiment. The assignments, the majority of which have not been available previously for this compound, are summarized in Table 2. The glycan primary structure was further substantiated by detection of the following dipolar interactions between glycosidically linked residues in the NOESY experiment: V-1/IV-3; IV-1/III-3; III-1/II-4; and II-1/I-4. Although no interglycosidic dipolar interactions were detected originating from the NeuAc residue, the NeuAcα2→3 linkage to terminal β-Gal was confirmed by the diagnostic positions observed for V-3, V-4, and A-3ax (*26*, *27*).

The complex of resonances corresponding to the major ceramide component indicated that it is composed of sphing-4-enine and non-α-hydroxylated fatty acids (*26*, *32*). Only a small amount of fatty acid unsaturation is indicated by the low amplitudes of the cis-vinyl proton signal at 5.320 ppm and the allyl proton signal at 1.976 ppm. The presence of a ceramide component containing α-hydroxylated fatty acids is indicated by the observation of additional β-Glc H-1 and H-2 resonances at 4.215 and 3.026 ppm, respectively (*27*).

Permethylation and ESI-MS. Following permethylation, a portion of the material was subjected to analysis by electrospray ionization mass spectrometry in the positive ion mode. Single quadrupole analysis at high orifice-to-skimmer potentials (150−200 V) gave abundant singly charged [M·Na]$^+$ and doubly charged [M·2Na]$^{2+}$ pseudomolecular ion species. The overall abundances, as well as the ratio, of these species depended on the potential used, although they were not always strictly reproducible even at a given potential due to the influence of other variables. High potentials resulted in significant and useful glycosidic fragmentation, yielding ions of the B- and [C·Na]-type, along with some of the [B·Na]- and [Y/C·Na]-type ions[2] normally seen under ESI-MS/CID-MS conditions. An additional set of ions corresponding to [C·Na + CH_2] were observed for a number of glycosidic cleavages; the precise origin of these fragments will be discussed elsewhere.[3]

Scheme 1: Prominent Pseudomolecular Ions and Fragmentation of Permethylated Hexaglycosylceramide in ESI-MS at High Orifice-to-Skimmer Potentials[a]

fa	Cer	MW	M•Na$^+$	M•2Na^{2+}
16:0	548	2001	[2024]	[[1023.5]]
18:0	576	2029	[2052]	[[1037.5]]
20:0	604	2057	[2080]	[[1051.5]]
22:0	632	2085	[2108]	[[1065.5]]
24:0	660	2113	[2136]	[[1079.5]]
26:0	688	2141	[2164]	[[1093.5]]
h22:0	662	2115	[2138]	[[1080.5]]
h24:0	690	2143	[2166]	[[1094.5]]



[a] Key: [ ] = monosodiated monocation; [ ]* = monosodiated monocation + 14 u; [[ ]] = disodiated dication. All values are nominal monoisotopic masses. Fa = fatty acyl group.

The monosodiated pseudomolecular ion series (see Figure 5; Scheme 1) corresponded to a glycan formula NeuAc·Hex$_4$·HexNAc attached to ceramides consisting of d18:1 sphingosine in combination with fatty acids 16:0−24:0, with 22:0 and 24:0 predominating. The presence of significant amounts of h22:0 and h24:0 fatty acids was also apparent from pseudomolecular ions 30 u higher in mass (in the former case, the ion at *m*/*z* 2138 is poorly resolved from that at *m*/*z* 2136). The fragmentation clearly supports the linear glycan sequence proposed for the ganglioside, as shown in Scheme 1. Although the lack of an observable B_2 ion at *m*/*z* 580 means that the NeuAc residue cannot be definitively placed on the terminal Hex on the basis of this series alone, the combination of internal glycan ions [Y_5/C_6·Na] (*m*/*z* 1102) and [Y_4/C_6·Na] (*m*/*z* 898) is supportive. However, the latter ion is of rather low abundance. ESI-MS/CID-MS experiments, as described below, were more definitive.

Triple quadrupole ESI-MS/CID-MS experiments were carried out under conditions maximizing the yield of disodiated doubly charged pseudomolecular ions. The most abundant of these were selected by Q_1 and collisionally activated in Q_2 while scanning Q_3. The results of three of these experiments are reproduced in Figure 6. The spectra are characterized by an abundance of glycosidic cleavage fragments of the [B·Na]-, [C·Na]-, [Y·Na]- [Y/B·Na]-, and [Y/C·Na]-types (see Scheme 2). Of particular significance is the abundant ion *m*/*z* 620, corresponding to the [C_2·Na] fragment which clearly shows the attachment of NeuAc to the terminal Hex residue.

Linkage Analysis by GC-MS. The remainder of the permethylated ganglioside was depolymerized, reduced, and acetylated according to standard procedures, and the resulting partially methylated alditol acetates (PMAAs) analyzed by

[2] The nomenclature of Domon and Costello (*33*) is used here.

[3] Levery, S. B. (manuscript in preparation).

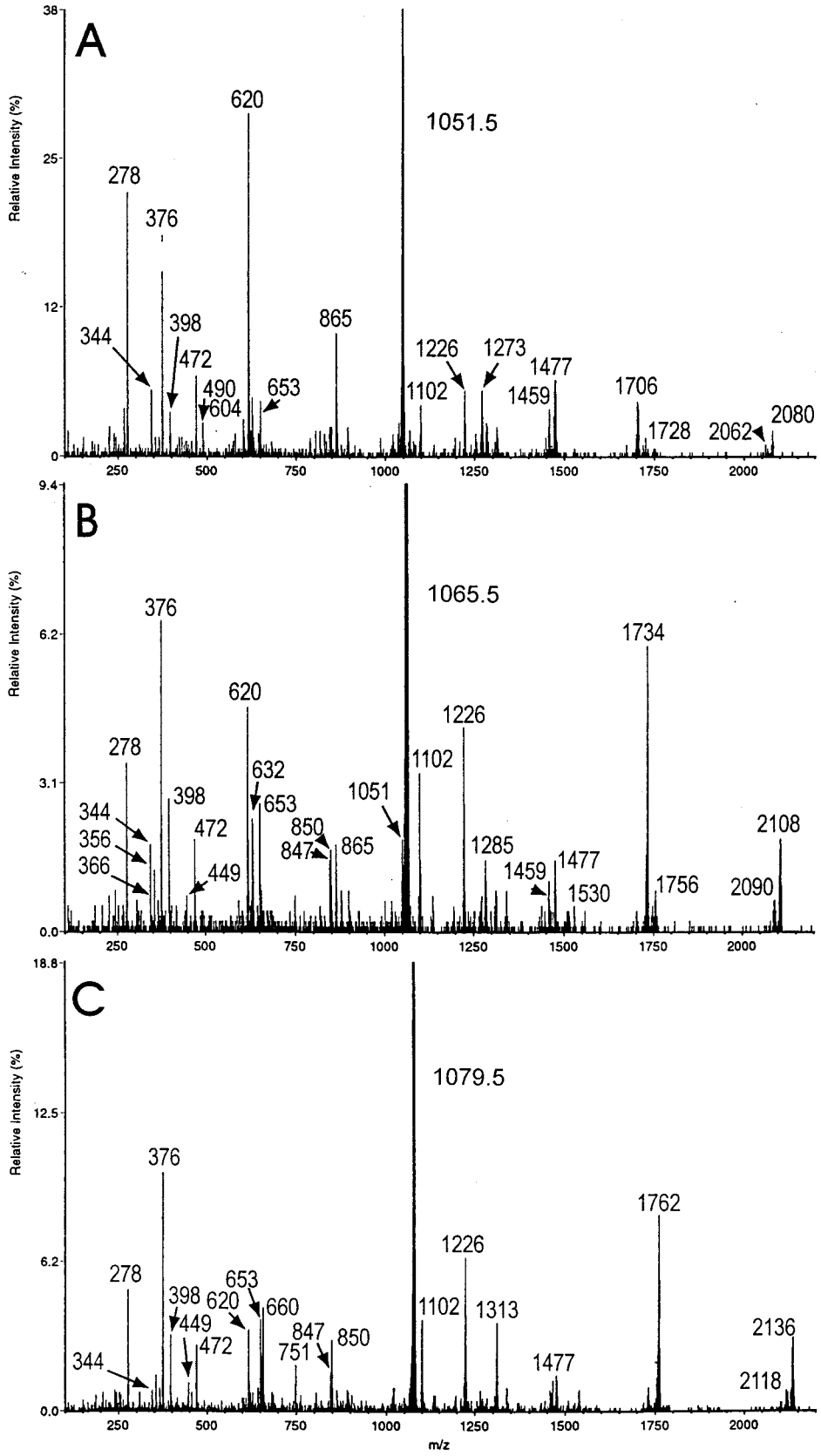
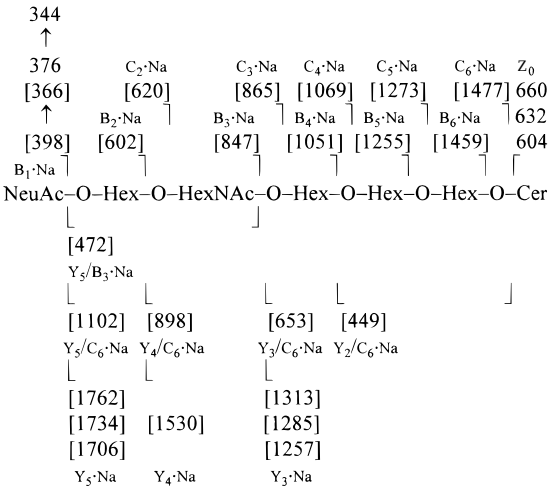


FIGURE 6: Product ion spectra from ESI-MS/CID-MS (orifice-to-skimmer potential = 120 V) of selected doubly charged disodiated pseudomolecular ions of permethylated monosialosyl ganglioside from human kidney. The orifice-to-skimmer potential was lowered to increase abundance of doubly charged disodiated pseudomolecular ions. Panel A, Q_1 selection of *m/z* 1051.5. Panel B, Q_1 selection of *m/z* 1065.5. Panel C, Q_1 selection of *m/z* 1079.5. Relevant fragments are designated by nominal monoisotopic mass (see Scheme 2).

GC-EI-MS. PMAAs identified by their retention times and characteristic EI spectra were 2,3,6-tri-O-Me-Gal (→4Gal); 2,4,6-tri-O-Me-Gal (→3Gal); 2,3,6-tri-O-Me-Glc (→4Glc); and 4,6-di-O-Me-GalNAcMe (→3GalNAc). Detection of

Scheme 2: Fragmentation of Q_1-Selected Doubly Charged Disodiated Pseudomolecular Ions $M{\cdot}2Na^{2+}$ of Permethylated Hexaglycosylceramide in ESI-MS/CID-MS[a]

fa	$M{\bullet}2Na^{2+}$	$M{\bullet}Na^{+}$	Cer
20:0	[[1051.5]]	[2080]	604
22:0	[[1065.5]]	[2108]	632
24:0	[[1079.5]]	[2136]	660



[a] Key: [ ] = monosodiated monocation. All values are nominal monoisotopic masses. Fa = fatty acyl group.

these derivatives confirmed unambiguously all of the linkages proposed on the basis of the NMR analysis, and, together with the residue sequence derived from ESI-MS data, confirms that the complete primary structure is $V^3NeuAcGb_5Cer$.

DISCUSSION

Globo-series glycosphingolipids are characterized by an internal Galα1→4Gal sequence and are found in the outer leaflet of the plasma membrane where they are exposed to the extracellular environment (*35*). The expression of globo-series glycolipids is determined by the P histo-blood group system (*1*, *2*) and plays an important role in the pathogenesis of UTI by mediating the attachment of *E. coli* to uroepithelial cells. Uropathogenic *E. coli* bind to P blood group antigens via *pap* gene encoded fimbrial adhesins, hence the name P fimbriae (*6*, *36*). In addition to the role of P blood group antigens in the pathogenesis of UTI, they have also been shown to act as receptors for verotoxins (*37*, *38*) and parvoviruses (*39*), and antibodies directed against some of these antigens (P and P^k) have been implicated in spontaneous abortion (*40*−*42*) as well as the rare autoimmune disorder paroxysmal cold hemoglobinuria (*43*).

SGG is a sialylated extended form of globo-series glycolipid first reported as a developmentally regulated antigen isolated and structurally characterized from the human teratocarcinoma cell line 2102Ep (*24*) and subsequently identified in chicken pectoral muscle (*44*). This antigen contains a terminal trisaccharide epitope (NeuAcα2→3Gal*β*1→3GalNAc) defined by a monoclonal antibody directed to murine stage specific embryonic antigen 4 (SSEA4). In addition to forming the terminal epitope of SGG, this trisaccharide is also found on extended ganglio-series glycolipids, i.e., GM_{1b}, GD_{1a}, and GT_{1b}, as well as on sialosyl galactosyl-A (*45*) and the monosialylated derivative of the T-antigen (Thomsen-Friedenreich antigen) present on O-linked glycoproteins.

In our previous study, glycosphingolipids were extracted from vaginal epithelial cells collected from women who were nonsecretors and secretors of ABO blood group antigens (*12*). A radiolabeled *E. coli* isolate (R45) bound to two glycolipids present in the extract derived from nonsecretors but not from secretors when separated on TLC plates. These glycolipids comigrated with SGG and DSGG standards and the higher migrating glycolipid (comigrating with SGG) stained positive with a monoclonal antibody that binds SGG. The presence of SGG in tissues of nonsecretors is consistent with the idea that an α2→3sialyltransferase preferentially sialylates the precursor galactosyl globoside in the absence of the α1→2fucosyltransferase encoded by the *Se* gene.

The data presented in this report describe for the first time the purification and unambiguous structural characterization of SGG from human kidney and support the hypothesis that SGG is an important physiological receptor for uropathogenic *E. coli* expressing *pap*-encoded adhesins. In a parallel study, we demonstrated that this human kidney derived SGG binds to wild-type uropathogenic *E. coli* strain R45 in addition to cloned *pap* adhesin-expressing *E. coli* strains representing all three classes of known P fimbrial adhesins (*13*). More important, all strains of *E. coli* assayed bind to SGG with high avidity when compared to closely related globo-series glycosphingolipids.

Earlier studies by Karr et al. (*47*) showed that *pap-2*-encoded P-fimbria purified from a pyelonephritic strain of *E. coli* bound to cryostat sections of human kidney and to human erythrocytes expressing the Luke (LKE) antigen (defined by anti-LKE serum) and that binding to kidney sections could be inhibited by preincubation with a monoclonal antibody to SSEA4 (defined by MAb MC813−70). They concluded that the receptor for the *pap-2* encoded P-fimbria was LKE on human erythrocytes and SSEA4 on human kidney. Although it has been suggested that LKE and SSEA4 are identical antigens, the relationship is limited to a common determinant recognized by both anti-LKE serum and MAb MC813−70. Regardless of the fact that this determinant is found on SGG, the presence of this glycolipid in human kidney or any other normal human tissue has until now only been speculative. A recent study described the fine specificity of a monoclonal antibody directed to DSGG (*48*). This MAb is able to discriminate between a common branched tetrasaccharide epitope (NeuAcα2→3Gal*β*1→3{NeuAcα2→6GalNAc) found on DSGG, GD1α, and a common mucin-type epitope widely distributed on glycoproteins such as glycophorin A. The differential antibody-binding ability of this structure is thought to be dependent on its carrier glycoconjugate and branched characteristics. Monoclonal antibodies specific for globo-series ABH antigens (*49*, *50*) as well as SGG are unavailable, and the difficulty in generating specific MAbs to these structures may be due to their linear characteristics. Unfortunately, until such MAbs are available, studies involving their tissue distribution will be extremely difficult. The results of this investigation clearly illustrate the presence of SGG in human kidney and may suggest a chemical basis for the increased risk of UTI in nonsecretors.

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