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SHORT COMMUNICATION

Structural characterization of blood group A glycosphingolipids recognized by the antibody 3G9-A

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In this study, the antibody 3G9-A was assayed for activity against human erythrocyte glycosphingolipids. The antibody was found to recognize glycosphingolipid components from blood group A erythrocytes but not glycosphingolipids from blood group B or O erythrocytes. Subsequent investigation revealed that the glycosphingolipid components recognized by the antibody were also recognized by a blood group A specific monoclonal antibody. The structures of two of the isolated active glycosphingolipid components were structurally characterized using proton nuclear magnetic resonance (^{1}H NMR) and gas chromatography-mass spectrometry (GC-MS) techniques and were found to consist of two blood group A glycosphingolipids; the type 2 chain A^b and type 3 chain A^a glycosphingolipids. Subsequent analysis of the remaining active components by GC-MS and immunostaining techniques revealed that all of the active components were blood group A glycosphingolipids. Furthermore, structural studies of the active components suggested that the epitope of the antibody consisted of the group A trisaccharide, GalNAcα1,3(Fucα1,2)Gal.

Keywords: Blood group A glycosphingolipids, monoclonal antibody, NMR, mass spectrometry

Abbreviations: GC-MS, gas chromatography-mass spectrometry; ^{1}H NMR, proton nuclear magnetic resonance; Gal, D-galactose; Glc, D-glucose; Fuc, L-fucose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; Cer, ceramide; mAb, monoclonal antibody; BSA, bovine serum albumin; PBS, phosphate buffered saline; FID, free induction decay; PMAA, partially methylated alditol acetates.

Introduction

Blood group ABH antigens are carried by membrane bound glycoproteins and glycolipids of human erythrocytes. The pioneering work of Hakomori and coworkers [1–3] has demonstrated that these antigens are defined by a complex array of carbohydrate chains attached to these glycoconjugates, including highly branched oligosaccharide chains. A set of anti-A monoclonal antibodies has served as a valuable tool for the detection and purification of the various A antigenic glycosphingolipids that have defined the structural diversity of this epitope (see Fig. 1 for structures). We have identified an antibody that binds to blood group A glycosphingolipids and provide an initial characterization of its specificity. A complete structural analysis of some of the glycosphingolipids recognized by this antibody has been done and the structural assignments support those presented by Hakomori and coworkers [4–8]. The results of our studies demonstrate that antibody 3G9-A binds to a wide array of blood group A glycosphingolipids and therefore, is a useful reagent for detecting most if not all blood group A antigens.

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Materials and methods

Preparation of 3G9 antibody (ascites fluid preparation) has been reported [9, 10]. Initial characterization of the 3G9 antibody resulted in the identification of an antibody specificity for a bacterial lipooligosaccharide (LOS) [9–11]. Subsequently, it was determined that the 3G9

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Type 1 Chain:

A[a]

```
GalNAcα1→3Galβ1→3GlcNAcβ1→3Galβ1→4Glc
              2
              ↑
          Fucα1
```

Type 2 Chain:

A[a]

```
GalNAcα1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc
              2
              ↑
          Fucα1
```

A[b]

```
GalNAcα1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc
              2
              ↑
          Fucα1
```

norA[c]

```
GalNAcα1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc
              2
              ↑
          Fucα1
```

isoA[c]

```
GalNAcα1→3Galβ1→4GlcNAcβ1
              2           ↓
              ↑           3
          Fucα1           Galβ1→4GlcNAcβ1→3Galβ1→4Glc
                          6
                          ↑
GalNAcα1→3Galβ1→4GlcNAcβ1
              2
              ↑
          Fucα1
```

A[d]

```
GalNAcα1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1
              2                            ↓
              ↑                            3
          Fucα1                            Galβ1→4GlcNAcβ1→3Galβ1→4Glc
                                           6
                                           ↑
                 GalNAcα1→3Galβ1→4GlcNAcβ1
                               2
                               ↑
                           Fucα1
```

Type 3 Chain:

A[a] GalNAcα1→3Galβ1→3GalNAcα1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc
(Fucα1→2 on each Galβ1 of the first and second Galβ1→ residues)

```
A^a   GalNAcα1→3Galβ1→3GalNAcα1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc
                 2                   2
                 ↑                   ↑
               Fucα1               Fucα1
```

A[b]

```
A^b   GalNAcα1→3Galβ1→3GalNAcα1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc
                 2                   2
                 ↑                   ↑
               Fucα1               Fucα1
```

Type 4 Chain:

```
A^x   GalNAcα1→3Galβ1→3GalNAcβ1→3Galα1→4Galβ1→4Glc
                 2
                 ↑
               Fucα1
```

Figure 1. Blood group A oligosaccharides. Nomenclature for each of the blood group A oligosaccharides is given in bold type. Defining sequences: type 1 chain core structure, Galβ1,3GlcNAcβ-R; type 2 chain core structure, Galβ1-4GlcNAcβ1-R; type 3 chain core structure, Galβ1-3GalNAcα1-R; type 4 chain core structure, Galβ1-3GalNAcβ-R.

antibody also contained binding activity for human blood group A erythrocytes. Cross absorption of the antibody with LOS did not remove the blood group A erythrocyte binding activity and confirmed the independence of the two antibody specificities identified. Therefore, the antibody described in these studies is designated 3G9-A to distinguish it from the anti-LOS antibody, 3G9. The monoclonal antibody A581 (reacts with group A compounds with type 1, type 2 and type 3 core structures) was kindly provided by Hakon Leffler, University of California, San Francisco, California. Information on the methods for the glycolipid isolation and immuno-detection methods have been reported previously [12–14]. Selected 3G9-A positive glycolipid fractions that were determined to consist of more than one type of glycolipid were subsequently separated into their constituent glycolipids using preparative thin-layer chromatography [15] of acetylated glycosphingolipids. Information on the methods utilized for structural characterization are presented in the Figure captions and Tables.

Results and discussion

Isolation of glycosphingolipids recognized by the 3G9-A antibody

Preliminary TLC immunostaining of erythrocyte neutral glycosphingolipids with the 3G9-A antibody showed a prominent positively staining component in glycosphingolipids from blood group A erythrocytes, whereas the 3G9-A antibody demonstrated no affinity for glycosphingolipids from blood group B and O erythrocytes (not shown). To further characterize the specificity of the 3G9-A antibody, neutral glycosphingolipids from blood group A erythrocytes were separated by high-performance liquid chromatography (Fig. 2). Four 3G9-A reactive bands were detected and designated bands 1–4 based on their TLC migration pattern, with the lowest running glycosphingolipid being referred to as band 1 and the highest running glycosphingolipid designated as band 4 (Fig. 3a,b). Since the orcinol stain of the four 3G9-A reactive glycosphingolipids indicated that band 3 was the most abundant component, initial attempts to determine the structure recognized by the 3G9-A antibody were centred on characterizing the structure of band 3. Preliminary NMR analysis of band 3 suggested that it was composed of more than one glycosphingolipid. To confirm the presence of more than one glycosphingolipid component in the band 3 fraction, an aliquot of the band 3 fraction was acetylated and analysed by thin-layer chromatography. Figure 4 shows that the acetylated band 3 fraction was resolved into four distinct glycosphingolipid components. After confirming the presence of more than one glycosphingolipid component, the four acetylated glycosphingolipid components in the band 3 fraction were isolated by preparative TLC, deacetylated

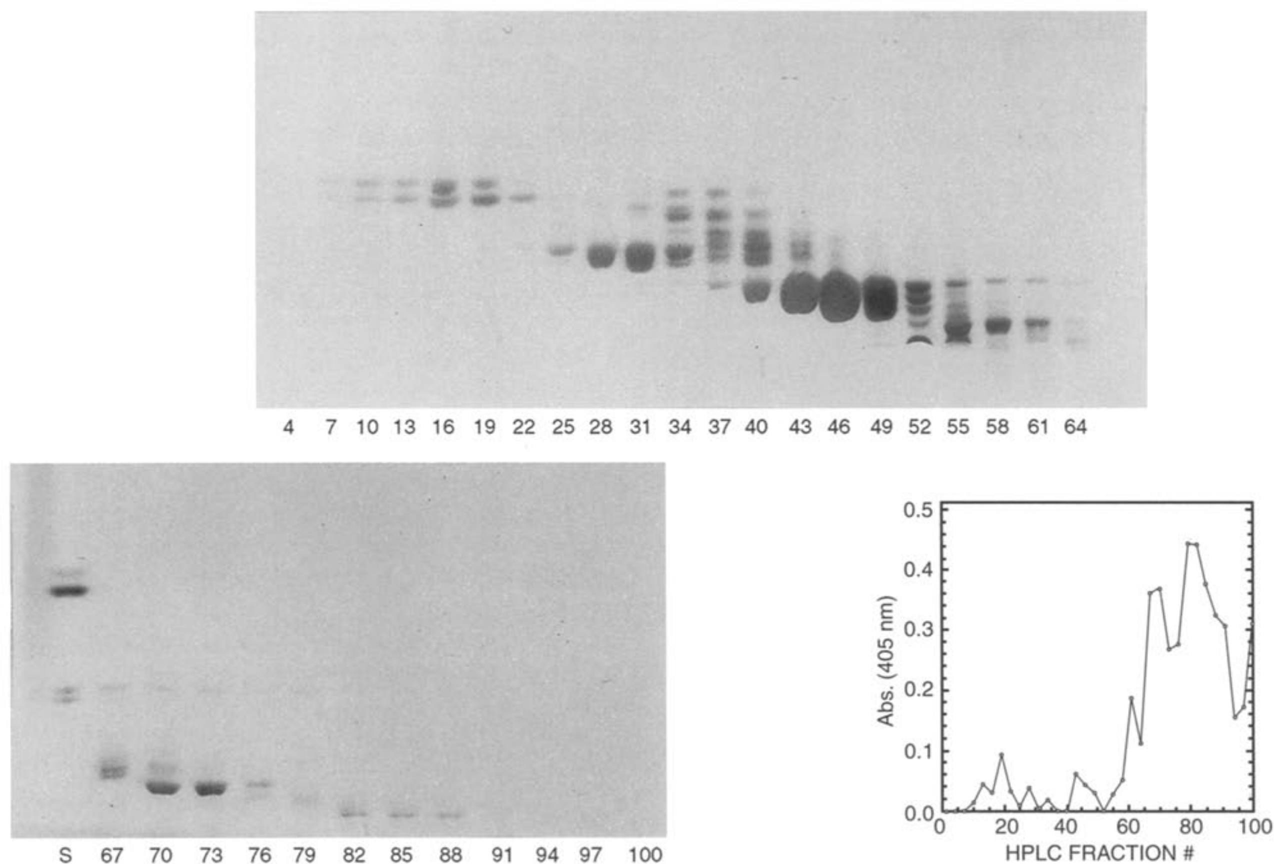

Figure 2. TLC and ELISA screening of HPLC fractions of neutral glycosphingolipids from blood group A erythrocytes. (A) Orcinol stained HPTLC plates of every third fraction from an HPLC separation of glycosphingolipids from blood group A erythrocytes. TLC plates were developed in a solvent tank containing chloroform:methanol:water (60:35:8, by vol). (B) Graphical representation of ELISA results from the screening of HPLC fractions containing glycosphingolipids from blood group A erythrocytes. The 3G9-A antibody was the primary antibody used in the assay.

and then structurally analysed. The four isolated glycosphingolipid components were designated band A, band B, band C and band D based on their migration as acetylated glycosphingolipids on the TLC plate (Fig. 4). All of the deacetylated components migrated to the same level on the TLC plate and were bound by the 3G9-A antibody.

Structural characterization of band 3 components

To further characterize the oligosaccharide structure of the 3G9-A reactive glycosphingolipid components, partially methylated alditol acetate (PMAA) derivatives of the 3G9-A reactive glycosphingolipids were prepared and analysed by gas chromatography-mass spectrometry (GC-MS). All of the 3G9-A reactive components were analysed in this manner with the exception of the band A component of band 3 which was not available in sufficient quantities for GC-MS analysis. A summary of the results of the GC-MS analysis for bands B, C and D is provided in Table 1. The results of the GC-MS analysis of bands B, C, and D indicate that all of the 3G9-A reactive glycosphingolipids contain the blood group A trisaccharide. The molar ratio and linkage position of the constituent sugars of band B are consistent with a type 2 chain A^b structure. On the basis of the GC-MS analysis of the PMAA derivatives of bands C and D it is concluded that both glycosphingolipids contain the same carbohydrate structures. The differences in the chromatographic properties of bands C and band D must therefore be due to differences in the ceramide moieties of the two glycosphingolipids. Furthermore, the R_f of the underivatized bands C and D on TLC plates using a chloroform:methanol:water (60:35:8, by vol) solvent system in conjunction with the GC-MS derived molar ratios of the constituent sugar residues suggests that band C and D contain the number and type of sugars consistent with a type 3 chain A^a structure. The GC-MS

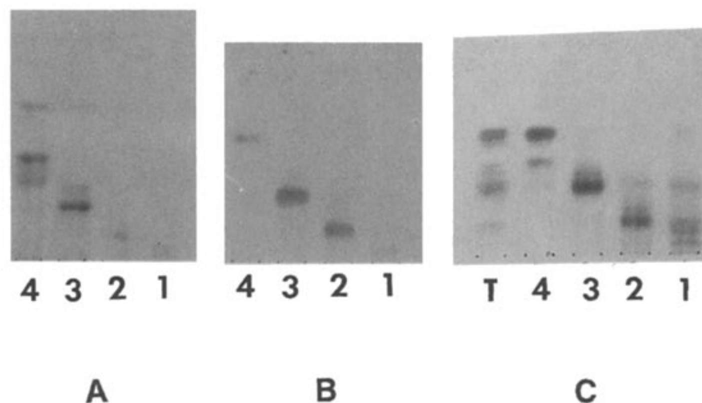

Figure 3. TLC analysis of four glycosphingolipid components for HPLC fractions containing neutral glycosphingolipids from human blood group A erythrocytes. Four glycosphingolipid components were obtained by combining selected HPLC fractions. The four components were then visualized on TLC plates by orcinol stain (A), immunostain using the 3G9-A antibody (B), and immunostain using the anti-blood group A monoclonal antibody A581 (C). Lane T, total neutral glycosphingolipids from blood group A erythrocytes.

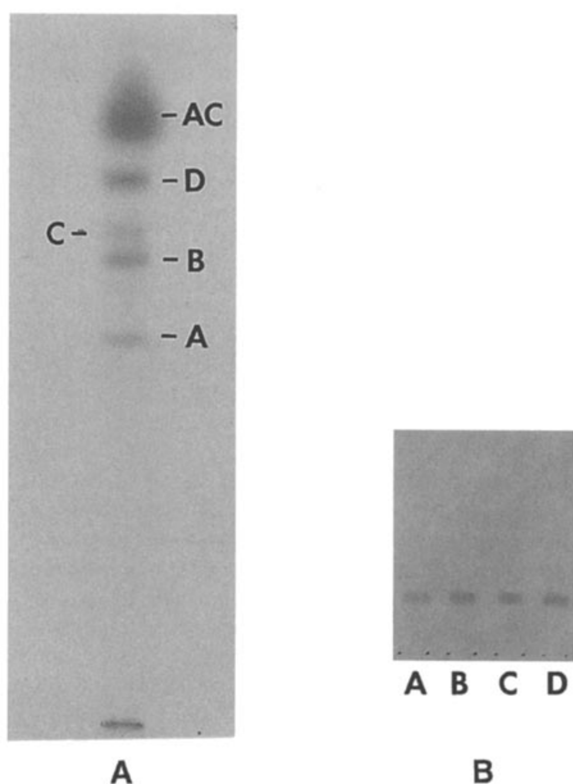

Figure 4. Separation of acetylated band 3 components and recognition of band 3 component glycosphingolipids by the 3G9-A antibody. A, Acetylated band 3 components were separated by thin-layer chromatography using a solvent system of 1,2-dichloroethane:acetone:water (40:60:2.5, by vol) and then visualized following Orcinol staining. The nomenclature assigned to the four components is given adjacent to each component (bands A–D). AC, acetylation reagents. B, The four band 3 component glycosphingolipids designated bands A–D were immunostained using the 3G9-A antibody. The TLC plate was developed in chloroform: methanol:water (60:35:8, by vol). Lane A, band A; lane B, band B; lane C, band C; lane D, band D.

Table 1. GC-MS derived sugar molar ratios found in band 3 components. Partially methylated alditol acetate (PMAA) derivatives of selected glycolipids were analysed by gas chromatography-mass spectrometry (GC-MS). Methylation was accomplished by a modification of a procedure described by Larson *et al.* [20]. The permethylated glycolipid was hydrolyzed with 2 M trifluoroacetic (100 °C for 6 h). The component sugar residues were subsequently reduced with a 5% (w/v) solution of sodium borodeuteride in methyl sulfoxide and water (40 °C for 90 min), and acetylated in 1-methyl imidazole and acetic anhydride. Analysis of the PMAA derivatives was performed using a Varian 3400 gas chromatograph with an OV-225 bonded phase fused silica capillary column (Anspec Inc., USA) linked to a Finnigan MAT ion-trap mass spectrometer using methane chemical ionization.

PMAA derivative	*3G9-A-reactive glycolipid*[a]		
	Band B	*Band C*	*Band D*
2,3,4-tri-*O*-Me-Fuc	0.8	1.5	2.3
2,4,6-tri-*O*-Me-Gal	1.4	1.3	3.0[b]
2,3,6-tri-*O*-Me-Glc	0.9	1.0	0.9
4,6-di-*O*-Me-Gal	0.7	1.4	1.5
3,4,6-tri-*O*-Me-GalNAcMe	1.0	1.0	1.0
3,6-di-*O*-Me-GlcNAcMe	1.6	0.9	1.3
4,6-di-*O*-Me-GalNAcMe	ND[c]	0.4	0.4

[a]Molar ratios normalized based upon chromatogram peak areas relative to the non-reducing terminal GalNAc residue, (3,4,6-tri-*O*-Me-GalNAcMe), present in each of the band 3 components.
[b]Questionable molar ratio due to overlapping peaks in the chromatogram.
[c]ND, none detected.

and immunostain results of the isolated band 3 components indicated that band 3 consisted of a mixture of the type 2 chain A^b and type 3 chain A^a glycosphingolipids. An analysis of the ^{1}H NMR spectrum of band 3 provided further confirmation of the identity of the two glycosphingolipids (Figs 5 and 6) [4–8].

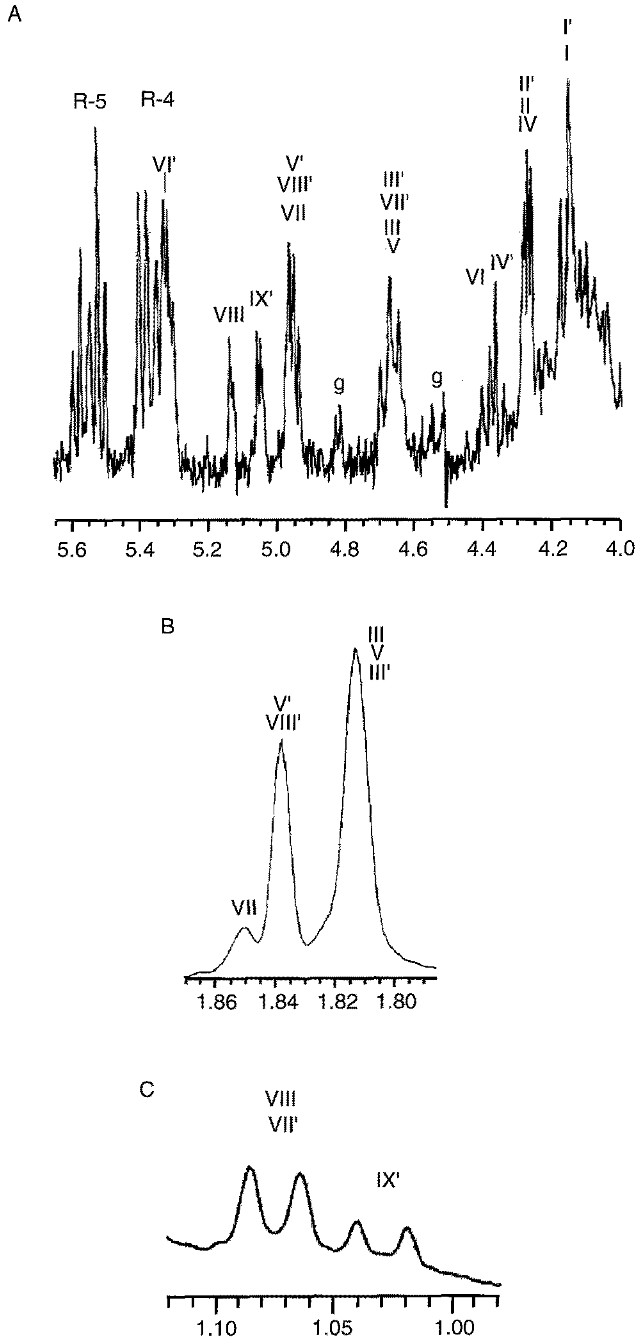

Figure 5. A, Downfield region from the ^{1}H NMR spectrum of band 3 containing anomeric proton resonances. R-4 and R-5, resonances associated with the vinylic protons in the sphingosine moiety of ceramide; g, resonances assigned to the anomeric protons of globoside (GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer). A small amount of globoside contaminant was noted in orcinol stains of all 3G9-A reactive bands not purified by preparative TLC. B, Upfield region from the ^{1}H NMR spectrum of band 3 showing the amino group methyl resonances associated with amino sugars. C, Upfield region from the ^{1}H NMR spectrum of band 3 showing the methyl H-6 resonances of fucose. Two thousand, two hundred and twenty four free induction decays were acquired on a 1 mg deuterium-exchanged sample of band 3 dissolved in 500 μl of dimethyl-d_6 sulfoxide with 0.5% D_2O. The acquisition was carried out at a temperature of 323° K. Roman numerals provide peak assignments for the anomeric protons, the methyl protons of the amino sugars and the fucose methyl protons based upon the structure of the type 2 chain A^b and type 3 chain A^a glycosphingolipids (refer to Fig. 6). Peak assignments were established by comparison with published chemical shift values observed in the ^{1}H NMR spectra of the respective group A glycosphingolipids acquired under similar conditions [4, 5]. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded with a General Electric QE-300 (300 MHz operating frequency) equipped with a General Electric GE 1280 computer and operating with quadrature detection. Between 2000 and 3000 free induction decays (FIDs) were collected per spectrum with presaturation of the water signal. The sweep width was 4 kHz collected over a 16 K data-set to give an acquisition time of 2.048 s and a digital resolution of 0.488 Hz per pt. A delay time of 1.00 s was added between the acquisition time and the beginning of the next pulse sequence. The resultant FID was processed by Fourier transformation. Proposed identity and associated anomerity of component sugars was based on the published peak resonances of glycosphingolipids analysed under similar conditions [4–7, 16–19].

Recognition of 3G9-A reactive glycosphingolipids by a blood group A-specific monoclonal antibody

The fact that four immunoreactive glycosphingolipid bands were observed in immunostains of blood group A erythrocytes but no reactive components were observed in immunostains of blood group O or B glycosphingolipids hinted that the reactive glycosphingolipids could be blood group A glycosphingolipids. This suggestion was strengthened by the GC-MS and NMR analysis of the isolated components of band 3 which confirmed their identity as blood group A glycosphingolipids. To further substantiate the identification of the band 3 components and confirm that the remaining 3G9-A reactive bands contained the blood group A epitope bands 1–4 were immunostained using the A581 monoclonal antibody which recognizes glycosphingolipids bearing the group A trisaccharide GalNAcα1-3(Fucα1-2)Galβ1. The results of the immunostaining are provided in Fig. 3C. The immunostaining results not only confirm that all of the 3G9-A reactive components are blood group A glycosphingolipids but they also clearly demonstrate that each

A)

```
 VII          VI            V            IV           III         II        I      R
GalNAcα1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer
              2
              ↑
            Fucα1
            VIII
```

B)

```
 VIII'        VII'         V'            IV'          III'        II'       I'     R
GalNAcα1→3Galβ1→3GalNAcα1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer
              2                          2
              ↑                          ↑
            Fucα1                      Fucα1
             IX'                        VI'
```

Figure 6. Structures and residue designations used in ^{1}H NMR analysis of band 3. A, The structure of type 2 chain A^b glycosphingolipid. B, The structure of type 3 chain A^a glycosphingolipid. Roman numerals signify sugar residues in the two glycosphingolipid structures. R, ceramide moiety of the glycosphingolipids.

Table 2. GC-MS analysis of PMAA derivatives from 3G9-A reactive components.

PMAA derivative	*3G9-A reactive component*			
	Band 1	*Band 2*	*Band 3*	*Band 4*
2,3,4-tri-*O*-Me-Fuc	+	+	+	+
2,4,6-tri-*O*-Me-Gal	+	+	+	+
2,3,6-tri-*O*-Me-Gal[a]	+	+	+	+
2,3,6-tri-*O*-Me-Glc	+	+	+	+
4,6-di-*O*-Me-Gal	+	+	+	+
2,4-di-*O*-Me-Gal	+	+		
2,3,6-tri-*O*-Me-GalNAcMe	+	+	+	+
3,6-di-*O*-Me-GlcNAcMe	+	+	+	+
4,6-di-*O*-Me-GalNAcMe		+	+	+

The presence of a particular PMAA derivative in a 3G9-A reactive glycolipid component is indicated by a plus sign.
[a]The 1,4-linked galactose derivative present in these glycosphingolipid samples is most likely due to the presence of a small amount of globoside, (GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer), contaminant that is noted in orcinol stains of all 3G9-A-reactive bands not purified by preparative TLC.

of the bands contain more than one glycosphingolipid with the blood group A epitope. To verify that all of the 3G9-A reactive glycosphingolipids contained the blood group A trisaccharide, PMAA derivatives of bands 1, 2, 3 and 4 were prepared and analysed by GC-MS in the same manner as previously described for the isolated components of band 3. The results of the GC-MS analysis are provided in Table 2. The GC-MS results clearly indicate that all of the 3G9-A reactive glycosphingolipids contain the terminal GalNAc, fucose, and 1,2,3-linked galactose residues that make up the blood group A trisaccharide.

Summary

A summary of the characteristics of the four 3G9-A reactive components is provided in Table 3. 3G9-A antibody was found to bind to a wide variety of blood group A glycosphingolipids (i.e. type 2 A^b, type 3 chain A^a, type 2 chain A^d). The fact that the 3G9-A antibody recognizes the blood group A trisaccharide linked in such radically different manners to different amino sugars suggests that the epitope recognized by the 3G9-A antibody probably does not extend beyond the blood group A trisaccharide itself. The differences in the spacial arrangements of the two tetrasaccharide structures virtually precludes the possibility that residues other than the group A trisaccharide participate in the binding of the antibody to the blood group A glycosphingolipids.

Acknowledgements

The 3G9 antibody was kindly provided by Peter A. Rice. This work was supported by grant AI21620 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

Table 3. Characteristics of 3G9-A reactive glycosphingolipid components.

Band Nomenclature	*Migration on HPTLC plate*[a]	*Predicted No. of sugars*[b]	*Glycosphingolipid components*[c]
1	1	>14	Type 2 chain A^d
2	2	10–12	Type 2 chain NorA^c and IsoA^c, Type 3 chain A^b
3	3	8–9	Type 2 chain A^b, Type 3 chain A^a
4	4	6–7	Type 4 chain A^x, Type 2 chain A^a

[a]Based on development in chloroform:methanol:water (60:35:8, by vol) solvent system. 1, *lowest migrating band; 4, highest migrating band.*
[b]Prediction based on characteristic R_f values associated with the development of glycosphingolipids on Silica Gel 60 HPTLC plates *using the solvent system described in (a).*
[c]Glycosphingolipid identification was based upon TLC immunostaining and GC-MS results, as well as predicted frequency of glycosphingolipid components in random donor units. Identification of type 2 chain A^b and type 3 chain A^a glycosphingolipids was also based on ^{1}H NMR studies of the isolated glycosphingolipids. For glycosphingolipid structures, refer to Fig. 1.

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