Glycoconjugate J (1990) 7:75-84

Detection of Gangliosides by Direct Binding of *Limax flavus* Agglutinin to Thin Layer Chromatograms

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Received July 13, 1989.

Key words: gangliosides, Limax flavus lectin, sialic acid, thin layer chromatography, autoradiography

A simple and sensitive method for detecting gangliosides on TLC plates is described. Gangliosides are extracted by phase partition in chloroform/methanol, developed on TLC plates in chloroform/methanol/0.25% aqueous KCl (5/4/1 by vol) and identified by binding of ^{125}I-labelled, sialic acid-specific *Limax flavus* agglutinin (LFA) autoradiography and scanning densitometry. The detection limit of the method is below 1 ng (0.5 pmol) for GM3, GM1 and GT1b, and below 0.3 ng (0.2 pmol) for GM2 and GD1a. Binding of ^{125}I-LFA is not inhibited by 10^6-fold molar excess concentrations of *N*-acetylneuraminic acid or lactose but is decreased in a dose-dependent manner by either *N*-acetylneuraminyllactose or unlabelled lectin. Gangliosides were not detected after their treatment by *Clostridium perfringens* sialidase in the presence of taurocholic acid. Ten gangliosides were detected in human brain and seven in normal human serum. Extracts from 0.2 mg of brain and 20 µl of serum were sufficient for the detection of major gangliosides.

Gangliosides are distinguished from other glycosphingolipids by the presence of a characteristic carbohydrate constituent, sialic acid. This compound can be used for the detection of microgramme amounts of gangliosides by chemical methods, e.g. using resorcinol or thiobarbituric acid [1, 2]. The introduction in 1980 by Magnani *et al.* [3] of the overlay technique on TLC plates has facilitated the analysis of glycolipids [4, 5]. This approach has been used to identify glycolipids that bind toxins [3], monoclonal antibodies [6, 7], lectins [8] and cells [9, 10]. Another solid phase method now available for the detection of gangliosides is the enzyme-linked lectin assay (ELLA) [11, 12], which involves binding of

Abbreviations: LFA; *Limax flavus* agglutinin. ELLA; Enzyme Linked Lectin Assay. PIM; Poly(isobutyl methacrylate). PVP; Polyvinylpyrrolidone mol.wt. 40,000. PBS; Phosphate buffered saline. BSA; Bovine serum albumin.

Gangliosides are named according to the system of Svennerholm (1963) J Neurochem 10:613-23, as follows: GM3; NeuAcα2-3Galβ1-4GlcCer. GM2; GalNAcβ1-4(NeuAcα2-3)Galβ1-4GlcCer. GM1; Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4GlcCer. GD1a; NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4GlcCer. GT1b; NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4GlcCer. GQ1b; NeuAcα2-8NeuAcα2-3Galβ1-3GalNAc(NeuAcα2-8NeuAcα2-3)Galβ1-4GlcCer.

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lectin to glycolipids immobilized on a plastic surface [13]. In contrast to the overlay technique ELLA is not suitable for glycolipids mixtures, only for individual, purified components.

Lectins are useful tools for the detection, isolation and structural analysis of glycoproteins and glycolipids [14]. Sialic acid-binding lectins appear to be present mainly in invertebrates and, with two exceptions, *Triticum vulgaris* [15] and *Lactuca scariole* [16], are not found in plants. Three preferential sialic acid-binding lectins have been isolated as homogeneous proteins: limulin, carcinoscorpin and *Limax flavus* agglutinin. Limulin and carcinoscorpin have molecular weights above 400,000 and consist of at least 16 subunits which makes them difficult to handle. They also display an absolute requirement for Ca^{2+} to bind carbohydrates [17, 18]. *Limax flavus* agglutinin (LFA) has a molecular weight of 44,000 and consists of two equal-sized subunits. Of 20 sugars tested, only *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid inhibited the ability of LFA to haemagglutinate human erythrocytes [19]. We have therefore utilized ^{125}I-labelled LFA to detect gangliosides fractionated from lipid extracts of human brain and normal human sera on TLC plates.

Materials and Methods

Human brain, obtained through Dr. Colin Masters of the Mental Health Research Institute, Melbourne, was from the frontal lobe of a 70 year old male who died after a cerebrovascular accident. It had been stored at -70°C for two years before extraction.

Human blood samples were obtained from healthy volunteers; sera were separated by centrifugation and stored at -20°C for up to six months before extraction.

The lectin from *Limax flavus*, purchased from Calbiochem-Behring, Australia, was reconstituted in 0.05 M Tris, 0.1 M NaCl, pH 7.5, to a concentration of 1 mg/ml. Bovine brain gangliosides GM3, GM2, GD1a and GT1b, also from Calbiochem-Behring, were at least 98% pure by TLC. GM1 was from Supelco, USA. High performance, 20 x 20 cm TLC plates (silica gel 60, aluminium backed no. 5574) were from E. Merck AG, F.R.G. Poly(isobutyl methacrylate) (PIM) of high molecular weight was from Aldrich, USA. Acetylneuraminyl hydrolase (sialidase; EC 3.2.1.18) from *Clostridium perfringens* - Type VI, polyvinylpyrrolidone (PVP; molecular weight 40,000), lactose ("β-lactose", i.e. a mixture of 69% β-lactose and 31% α-lactose), *N*-acetylneuraminyllactose from bovine colostrum, bovine serum albumin (RIA grade), taurocholic acid and orcinol were from Sigma, USA. *N*-Acetylneuraminic acid was from Fluka, Switzerland. All other chemicals were of analytical grade.

LFA was iodinated by the chloramine T method [20]. The reaction mixture contained 10 µg of lectin, 28 µl of PBS, 500 µCi of Na^{125}I (carrier-free from Amersham, UK) and 1 µg of chloramine T in a total volume of 40 µl. After 10 minutes at 4°C the reaction was stopped by the addition of 2.4 µg of sodium metabisulfite. ^{125}I-LFA was separated from free ^{125}I in a column of Bio-Gel P6 (Bio-Rad, USA) in PBS-1% BSA. The specific activity of ^{125}I-LFA used in all experiments was 30 ± 5 µCi/µg. At this specific activity ^{125}I-LFA was stable for at least three weeks when stored at 4°C. However when its specific activity exceeded 35 µCi/µg it was stable for only 7-10 days and when above 50 µCi/µg was unable to bind gangliosides.

Extraction of gangliosides from brain and normal human sera was done according to the methods of Svennerholm and Fredman [21] with modifications described by Colman *et al.* [22]. Briefly, 1.0 g of tissue was disrupted in a glass-teflon hand homogenizer in 3 ml of water or 1 ml of serum was mixed with 2 ml of water. Following transfer into 8 ml of methanol, 4 ml of chloroform was added and the mixture shaken vigorously for 30 min at 22°C. After centrifugation at 2,000 x *g* for 30 min at 4°C, the pellet was discarded, the supernatant enriched with water (40:7 by vol), gently mixed and centrifuged at 2000 x *g* for 30 min at 4°C. The two phases were separated (interfacial fluff with the lower phase) and the lower phase was enriched with methanol (2:1 by vol), stirred and then combined with 0.01 M KCl (5:1 by vol). After mixing and centrifugation at 2,000 x *g* for 30 min at 4°C the upper phases were combined and both upper and lower phases dried by rotary evaporation. Residues were reconstituted in chloroform/methanol/water, 60/30/4.5 by vol, and left for 24 h. They were then centrifuged at 5,000 x *g* for 30 min at 4°C and the supernatants stored at -70°C prior to TLC.

Sialic acid was measured using Bial reagent with crystalline *N*-acetylneuraminic acid as a standard, as described elsewhere [23], and protein concentration in brain and serum extracts was measured by the Lowry method [24] using BSA as a standard. TLC plates were reduced to 10 x 10 cm or 8 x 8 cm (for sialidase experiments) and 10 µl samples were spotted 1 cm from the bottom of the plate, 1 cm from each side and 1 cm apart. Plates were developed in chloroform/methanol/0.25% aqueous KCl, 5/4/1 by vol. After drying, silica on the developed plates was prevented from detaching during further washing procedures by soaking for 90 s in 0.1% PIM in 95% hexane (dissolved overnight) and warm air-drying. Plates were then placed in plastic Petri dishes and soaked for 1 h at 4°C in PBS-PVP with continuous gentle agitation. PVP was used to block non-specific binding of labelled lectin. ^{125}I-Labelled lectin was added at 1-2 ng per cm^2 of plate in PBS-PVP (0.5 ml/cm^2). Plates were incubated for 1 h at 4°C in a covered dish, washed four times, 30 min each time, in cold PBS-PVP, dried and exposed to Amersham Hyperfilm-MP for 24-68 h at -70°C. Autoradiographs were scanned on an LKB Ultroscan XL densitometer and absorbances of gangliosides were corrected for background absorbance of the plate. Data were plotted semi-logarithmically using an Apple computer with Cricket Graph program version 1.3.

Glycolipids on TLC plates were treated with sialidase according to the method of Wenger and Wardell [25]. Standards (10 ng each), brain extracts (1.0 mg wet weight equivalent) or serum (50 µl equivalent) were developed on TLC plates in chloroform/methanol/0.25% aqueous KCl, 5/4/1 by vol, warm air-dried and soaked in 0.03% PIM in 95% hexane for 90 s, dried and washed in 0.05 M sodium acetate buffer with 0.2% sodium taurocholate pH 6.0 for 30 min. TLC plates were then transferred into square Petri dishes (10 x 10 cm) and overlaid with 40 ml of the same buffer with or without 10 U of *Clostridium perfringens* sialidase. Dishes were sealed with Parafilm and left for 40 h at 37°C with gentle orbital movement. The enzyme solution was removed and plates washed at 4°C in PBS-PVP 2 x 60 min before ^{125}I-LFA application.

Lactose, *N*-acetylneuraminic acid, *N*-acetylneuraminyllactose and LFA were used in competition studies with ^{125}I-LFA. TLC plates with standard gangliosides (20 ng or 2 ng each) were developed, fixed with PIM and soaked in PBS-PVP for 1 h. Sugars (10 µg/cm^2) were each dissolved in PBS-PVP (0.5 ml/cm^2) together with ^{125}I-LFA (1 ng/cm^2), applied on TLC

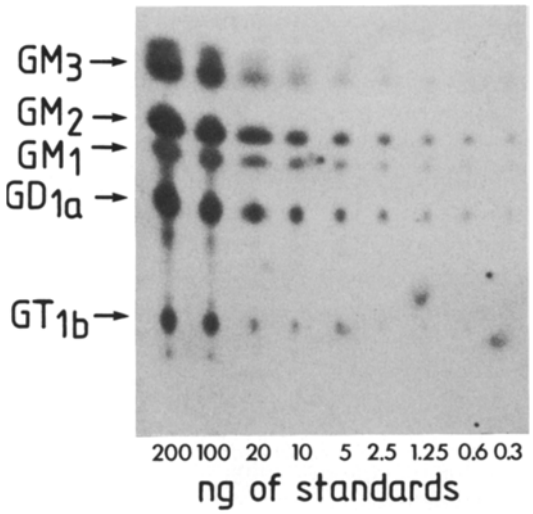

Figure 1. Autoradiogram of ^{125}I-LFA binding to ganglioside standards.

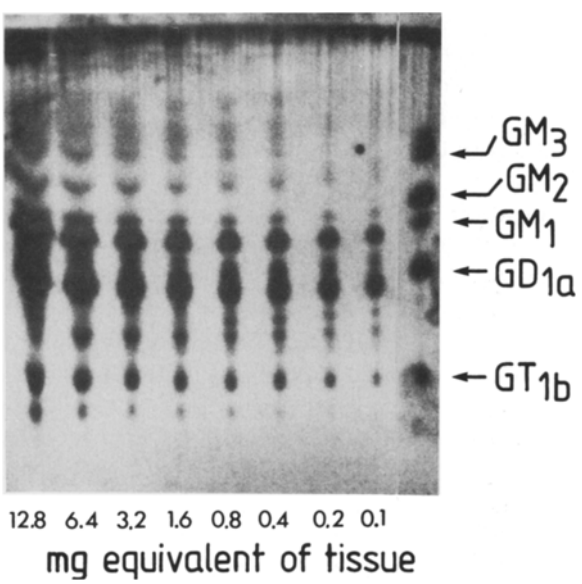

Figure 2. Autoradiogram of ^{125}I- LFA binding to gangliosides extracted from human brain

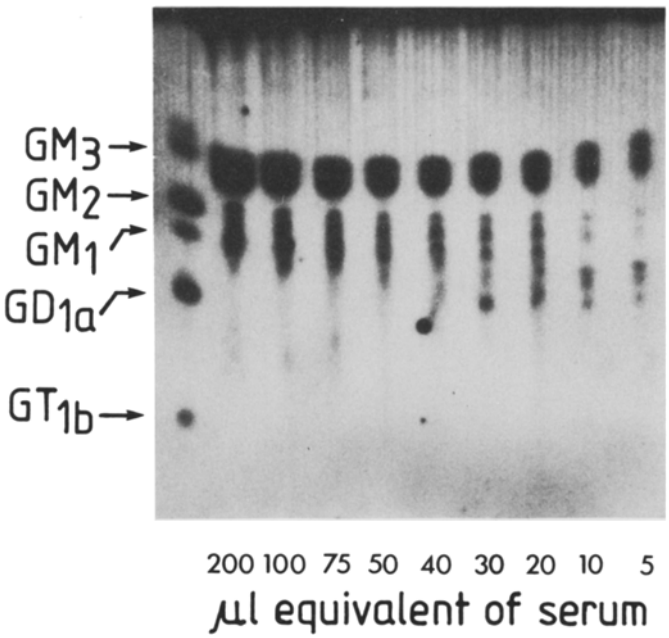

Figure 3. Autoradiogram of ^{125}I-LFA binding to gangliosides extracted from normal human sera.

plates and incubated for 60 min at 4°C with gentle agitation. Plates were washed 4 x 30 min in cold PBS-PVP, dried and autoradiographed. Binding of ^{125}I-LFA was also measured in the presence of a 10^4-fold molar excess of native LFA.

Results

^{125}I-LFA identified standard gangliosides, as well as gangliosides extracted from human brain and normal human sera (Figs. 1-3). The detection limit for gangliosides GM1, GM3, and GT1b was below 1 ng; GM2 and GD1a were clearly visible even at the lowest concentration tested, i.e. 0.3 ng. Resolution deteriorated and tailing was evident at concentrations of 100 ng and above (Fig. 1). Densitometric scanning of autoradiographs increased sensitivity even further (Fig. 4). A minimum of 10 different sialic acid-containing gangliosides was detected in human brain extracts. Polysialylated gangliosides GT1b and GQ1b were clearly visible down to 0.2 mg equivalent of wet brain tissue. In brain extracts, components migrating faster than GM3 were also evident (Fig. 2). In normal human sera seven major gangliosides were detected in the region of monosialylated and disialylated gangliosides, the main band being a doublet in the position of GM3 (Fig. 3).

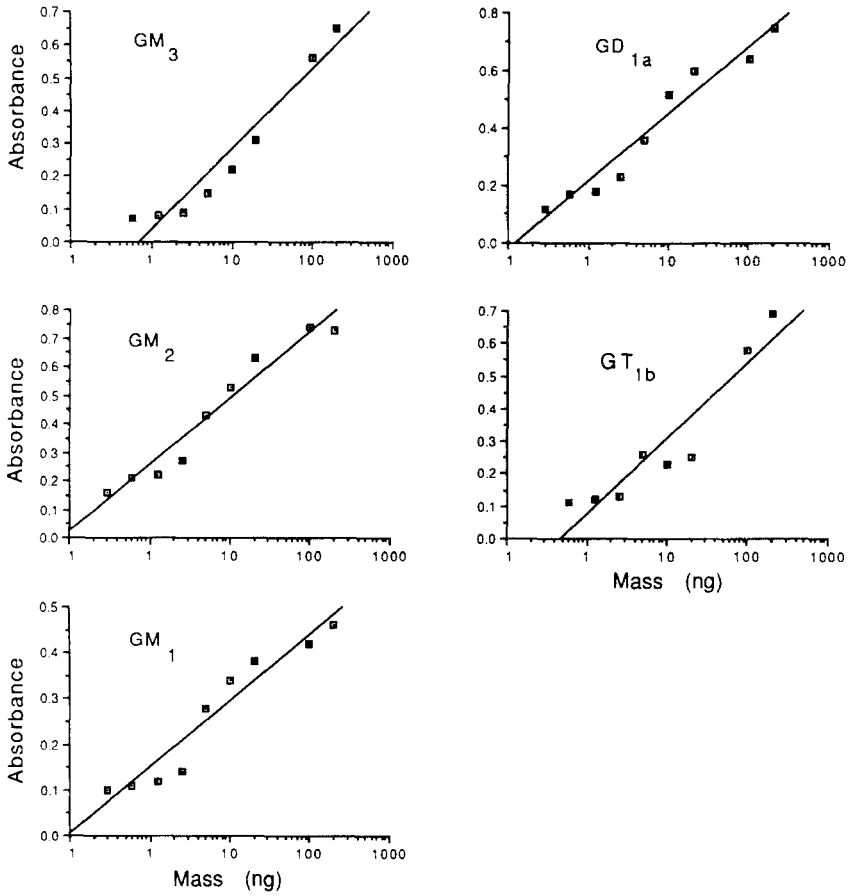

Figure 4. Relationship between log mass of each standard ganglioside and its densitometric absorbance value after TLC, detection by ^{125}I-LFA binding and autoradiography.

Binding of ^{125}I-LFA to standard gangliosides was abolished in the presence of unlabelled LFA (Fig. 5D). Neither *N*-acetylneuraminic acid nor lactose inhibited ^{125}I-LFA binding in the excess concentrations used, but *N*-acetylneuraminyllactose inhibited binding to the lowest concentration (2 ng) of standards (Fig. 5). The background activity in the presence of *N*-acetylneuraminic acid was consistently higher, presumably because of ^{125}I-LFA binding to *N*-acetylneuraminic acid non-specifically adsorbed to the plate (Fig 5A).

Gangliosides were not detected after treatment with *Clostridium perfringens* sialidase, even when applied at concentrations 10 times above the detection limit of the assay (Fig. 6).

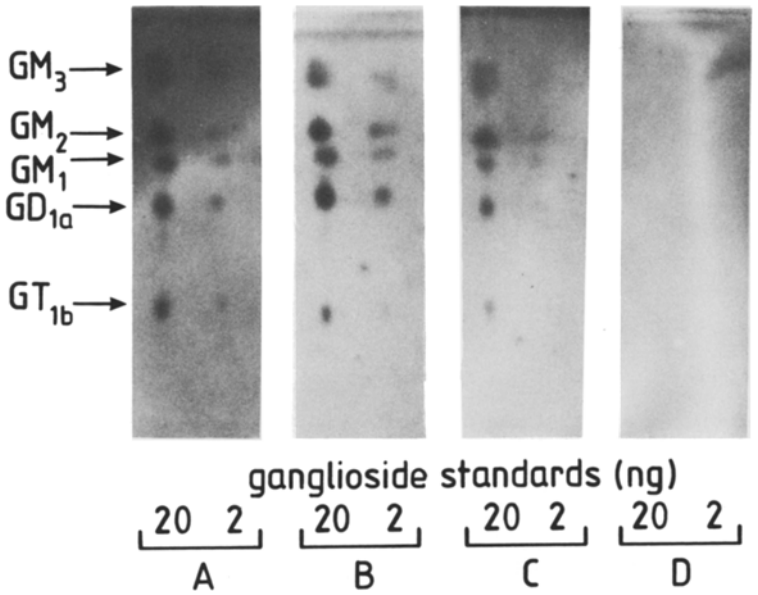

Figure 5. Binding of ^{125}I-LFA to standard gangliosides (20 ng and 2 ng) in the presence of *N*-acetylneuraminic acid (A), lactose (B), *N*-acetylneuraminyllactose (C) and unlabelled LFA (D).

The background on TLC plates was increased if the specific activity of ^{125}I-LFA exceeded 35 μCi/μg, the first wash was omitted, the reaction time with ^{125}I-LFA was increased to more than 2 h or when the concentration of PVP was lower than 1%. There was no difference in binding when the first wash was carried out for up to 18 h in 1% PBS-PVP at 4°C with or without sodium azide in concentrations of up to 0.02%. Substitution of 1% BSA for 1% PVP resulted in a five-fold decrease in sensitivity (data not shown).

Discussion

LFA through its binding to sialic acids on the oligosaccharide chain is a sensitive probe for detecting gangliosides with the TLC overlay technique.

The recovery of glycolipids in the single extraction step is at least 90% and, in the presence of 20% water, admixture by sialoglycoproteins and other proteins does not exceed 2% of total sialic acid content [26]. Reconstitution of gangliosides, after evaporation in chloroform/methanol/water diminishes the content of glycopeptides even further [21]. In our experiments proteins were not detected in supernatants of brain and serum extracts. The association properties of gangliosides are influenced by the medium, particularly by the presence of proteins and mono- and divalent cations.

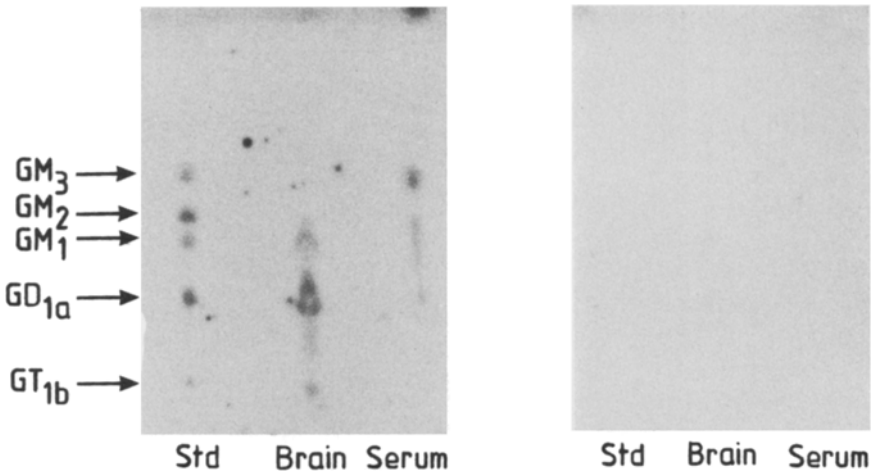

Figure 6. Binding of ^{125}I-LFA to standard gangliosides, and human brain and serum gangliosides, pre-treated without (left) or with (right) *Clostridium perfringens* sialidase.

BSA was avoided, because it caused a decrease in sensitivity, probably due to its interaction with gangliosides [27]. However salts, organic acids and simple saccharides present in the upper phase after extraction [28] did not prevent binding of ^{125}I-LFA. Unlabelled LFA and *N*-acetylneuraminyllactose decreased binding of ^{125}I-LFA to gangliosides, but lactose and *N*-acetylneuraminic acid were without effect.

Gangliosides were not detected after treatment of brain and serum extracts with *Clostridium perfringens* sialidase in the presence of taurocholic acid. These data demonstrate that LFA is specific for the detection of sialic acids on carbohydrate chains. The high sialidase concentration and long reaction time required may be explained by impaired access of the enzyme to sialic acid covered by a layer of plastic. If plates were not covered by PIM they detached from the aluminium backing after a few hours of incubation.

As little as 1 ng (0.5 pmol) of all standard gangliosides was detected by the ^{125}I-LFA binding method. This represents a sensitivity three orders of magnitude greater than achieved with the traditional resorcinol and thiobarbituric acid methods for sialic acid [1, 2]. The overlay techniques with monoclonal antibodies or cells detect 1-200 pmol of gangliosides [4, 10]; binding of ^{125}I-labelled galactose or *N*-acetylgalactosamine-specific lectins, as described by Smith [8], detects 100 pmol of neutral glycolipids. The variation in binding of ^{125}I-LFA to different ganglioside standards could be due to structural differences between ganglioside molecules. Moreover, GD1a and GT1b were not homogeneous and GM3 was found to

migrate as a doublet. The gangliosides that were detected with the highest sensitivity have the highest micellar charge [29]. This suggests that charge is an important determinant of LFA binding in aqueous solutions. GT1b which has twice as much sialic acid as GM2 (on a weight basis) bound ^{125}I-LFA weakly compared to the other standards. However, GT1b was also the only ganglioside in which two sialic acids are bound to each other, which may well result in steric hindrance to their interaction with LFA.

The analysis of gangliosides in human brain and serum illustrates the application of the method. The identification of 10 major gangliosides in brain is in agreement with the findings of others [30-32]. However, we also found three additional species above GM3. Although undefined, one may be GM4 and the other two could be lactones of monosialylated or disialylated gangliosides [33] either occurring naturally or as a result of isolation. Normal serum contained seven gangliosides. Only GT1b, found by others [34, 35] after much longer extraction and purification from a larger volumes of plasma, was not detected. There are differences in the migration patterns of gangliosides in higher versus lower concentrations of brain and serum extracts. This may be due to high concentrations of other lipids present in unpurified ganglioside preparations. The LFA binding method described here is a simple and sensitive assay for sialic acid-containing oligosaccharides in crude mixtures. It is well-suited to the qualitative and quantitative detection of gangliosides in biological specimens.

Acknowledgements

This work was supported by grant from the National Health and Medical Research Council of Australia and by a grant from Apex-Diabetes Australia.

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