A novel pentaglycosylceramide in ostrich liver, IV₄-β-Gal-nLc₃Cer, with terminal Gal(1→4)Gal, a xenoepitope recognized by human natural antibodies

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Thin layer chromatograms of ostrich liver neutral glycosphingolipids were immunostained with human sera. In addition to the expected staining of the Forssman pentaglycosylceramide by some sera, more polar and less abundant unknown glycolipids could be stained. Among them, the shortest carbohydrate chain glycolipid was purified and structurally characterized by mass spectrometry, proton NMR and methylation analysis. It was a novel pentaglycosylceramide of the neolactoseries terminated with the Gal(1→4)Gal determinant which is not expressed in mammalian species. Human antibodies affinity-purified on a synthetic Gal(1→4)Gal(1→4)Glc-Sepharose column recognized the newly characterized Gal(1→4)Gal-terminated pentaglycosylceramide, and, in addition, longer chain glycolipids. Occurrence of antibodies directed at the Gal(1→4)Gal epitope was studied by ELISA on 108 human sera. Anti-Gal(1→4)Gal antibodies were predominantly IgM, and their distribution was similar to that of anti-Gal(α1→3)Gal and anti-Forssman IgMs. It was concluded that anti-Gal(1→4)Gal are natural antibodies, not previously identified in man. They can be considered as xenotargets directed at species which express Gal(1→4)Gal-terminated carbohydrate chains.

Key words: Galβ4Gal/glycosphingolipid/xenoepitope/human natural antibodies/electrospray-ion trap mass spectrometry/1H-NMR

Introduction

Interest for heterophile antigens (xenoantigens), has been revived by the prospect of grafting porcine organs to man in order to alleviate the shortage of human organs (Auchincloss and Sachs, 1998). Xenoantigens are the target of natural antibodies present in human serum. The major porcine xenoantigen recognized by human natural antibodies is the Galα3Galβ1-4Gal epitope (Good et al., 1992; Galili, 1993; Sandrin et al., 1993), which is expressed in two types of structures, the afucoB epitope Galα3Galβ4GlcNAc (Jalali-Araghi and Machler, 1994; Samuelsson et al., 1994) and the Galα3Leα epitope (Bouhours et al., 1997, 1998b).

N-Glycolyneuraminic acid (HD antigen) (Hanganatziu, 1924; Deicher, 1926; Higashi et al., 1977) is the second heterophile antigen expressed in pig. Although the HD antigen is not expressed in man, the occurrence of preformed anti-HD antibodies is rare, but HD antigen is known to trigger a potent immune reaction.

Ostrich was interesting to study as an alternative to pig for experimental xenotransplantation, because, as a bird, it does not express the Galα3Gal epitope or the HD antigen. However, it has been found that ostrich tissues express the Forssman pentaglycosylceramide (Bouhours et al., 1999), which is the third known heterophile antigen (Forssman, 1911; Siddiqui and Hakomori, 1971; Stellner et al., 1973; Kano et al., 1984).

As the occurrence of natural anti-Forssman antibodies in human serum (Young et al., 1979; Strokan et al., 1998) is not as well documented as that of anti-Galα3Gal antibodies (Galili, 1993; Sandrin et al., 1993; Parker et al., 1994; McKane et al., 1998), the study of the anti-Forssman reactivity of human serum was reinvestigated by ELISA using purified Forssman antigen, and immunostaining of chromatograms of neutral glycolipids of ostrich liver. Surprisingly, it was found upon chromatogram-immunostaining that some sera were reactive at high dilution with glycolipids more polar and less abundant than the Forssman pentaglycosylceramide. One of these glycolipids was isolated from ostrich liver and characterized as Galβ4Galβ4GlcNAcβ3Galβ4GlcβCer. This is a novel structure related to blood group active glycolipids and presenting at the non-reducing end a determinant which is unknown in man and in mammals. Glycolipids with more than five sugar residues were also recognized by affinity-purified human anti-Galβ4Gal antibodies, but were not characterized in the present work. As the Galβ4Gal disaccharide appeared as a potential xenoantigen, the occurrence of human antibodies directed at this epitope was investigated by ELISA. It was found that anti-Galβ4Gal antibodies are mainly IgM and occur in a large population according to a distribution similar to that of natural anti-Galα3Gal and anti-Forssman antibodies.

Results

Chromatogram-immunostaining of ostrich liver neutral glycolipids with human serum IgM

It has been shown that ostrich liver chiefly expresses three neutral glycosphingolipids, the mono- and dihexosylceramides
and the Forssman pentaglycosylceramide F-5 (Figure 1A, lane 2) (Bouhours et al., 1999). Random human sera were used for immunostaining of thin layer chromatograms of ostrich liver glycolipids in order to evaluate their IgM anti-Forssman reactivity.

Some sera immunostained F-5, whereas other did not. Surprisingly, glycolipids more polar than F-5 were occasionally stained, with concomitant staining of F-5 (Figure 1B, lane 3) or without it (Figure 1C, lanes 5–6). These glycolipids were hardly detected by chemical visualization compared to F-5 (Figure 1A, lane 2). One of them, termed GL-5 before structural characterization, migrated at the same level as the Galα3Gal-terminated pentaglycosylceramide (afucoB-5) of porcine kidney (Figure 1A). However, it was not reactive with hen anti-Galα3Gal antibodies (Bouhours et al., 1998a), thus confirming the lack of expression of this epitope in birds. GL-5 accounted for 0.5% of the neutral glycolipid content of ostrich liver (0.5 µmol per liver), that is about one tenth of the F-5 contribution (Bouhours et al., 1999).

Structural characterization of GL-5 in ostrich liver

Although GL-5 was present in very low amount in the liver glycolipid extract, it was purified by preparative thin layer chromatography with a sufficient yield and quality for structural characterization.

Mass spectrometry. The ESI-IT-MS spectrum of GL-5 displayed a prominent molecular ion (m/z 1411.9) (Figure 2, upper panel). It was consistent with an oligosaccharide chain containing one HexNAc and four Hex linked to a ceramide containing C18 sphingosine (d18:1) and C16 nonhydroxylated saturated fatty acid (n16:0). The m/z value of this ion was identical with that of the afucoB-5 (IV3-α-Gal-nLc4Cer) of porcine kidney with the same ceramide. A peak at m/z 1539.8 could be interpreted as the same oligosaccharide chain linked to a ceramide portion containing C24 hydroxylated fatty acid (b24:0). Molecular ion m/z 1411.9 was submitted to collision-induced dissociation (Figure 2, middle panel, MS2) in order to obtain the carbohydrate sequence. Loss of the terminal sugar generated the Y4 ion (m/z 1249.7), consistent with a terminal Hex. Loss of the penultimate carbohydrate residue generated the Y3 ion m/z 1087.8, consistent with a terminal disaccharide HexOHex. The major ion generated by the fragmentation was the Y2 dihexosylceramide ion m/z 884.9 which arose from the additional loss of a HexNAc, indicating that the terminal trisaccharide was HexOHexOHexNAc which appeared as the B3 ion (m/z 349.9). The Y1 monohexosylceramide ion was observed (m/z 722.8) with the corresponding tetrasaccharide B4 ion (m/z 712.4) for HexOHexOHexNAcOHexHexOHexNAc which was manifested by B5 (m/z 874.5) and C5 (m/z 892.5) ions. Further fragmentation of the Y2 ion (Figure 2, lower panel, MS3) yielded the disaccharide ions HexOHex at the reducing end of the chain termed b2 (m/z 347.5) and c2 (m/z 365.8) by analogy with the B2 and C2 disaccharide ions at the nonreducing end of the oligosaccharide chain. Appearance of the Z0 ceramide ion (m/z 342.6) indicated the presence of a nonhydroxylated fatty acid (Bouhours et al., 1999). Although the chain length of the sphingoid base was not established by fragmentation of the Y2 ion, due to the lack of hydroxylation of the fatty acid (Bouhours et al., 1999), the only presence of sphingosine (d18:1) in other glycolipids of ostrich liver (Bouhours et al., 1999) was in favor of the same base in GL-5, consistent with the d18:1/n16:0 ceramide species.

Characterization of carbohydrate residues and linkages. GC analysis of the partially methylated alditol acetates of GL-5 yielded peaks for 2,3,4,6-tetra-O-Me-Gal (terminal Gal→1), 2,3,6-tri-O-Me-Gal→1) 2,3,6-tri-O-Me-Glc→1) 2,4,6-tri-O-Me-Gal→1) and 3,6-di-O-Me-Gal-NacMe→1) (Figure 3, upper trace). Together with the sequence established by MS analysis, these data were consistent with a Gal1–3/4Gal1–4GlcNAc1–3/4Gal1–4Glc1 chain. The presence of a galactose substituted on C-4 made GL-5 different from afucoB-5 which does not contain 4Gal-1-. However, methylation analysis could not determine which one of the two internal galactoses was substituted on C-4. The LiAlH4 reduction of the permethylated sugar chain is known to prevent cleavage of the internal GlcNAc-Gal linkage upon acid hydrolysis (Karlsson, 1974), and to give rise to a late eluting disaccharide peak (Karlsson and Larson, 1981). In the present analysis, the disaccharide GlcNAc-Gal peak was accompanied by the disappearance of the peak for 3Gal1→1) (Figure 3, lower trace). This result indicated that the GlcNAc was linked on C-3 of the previous Gal. Thus, the terminal disaccharide of GL-5 was Gal1–4Gal1–4Glc1. It was essential to determine the anomery of the linkage, in order to know whether the terminal disaccharide was Gal(α1–4Gal, the known blood group P determinant (Naiki et al., 1975), or Gal(β1–4Gal, a new epitope for glycolipids.

1H NMR spectroscopy. The main characteristic of the 400 MHz spectrum of GL-5 (Figure 4) was the lack of α-anomeric proton signal, and the presence of five β-anomeric proton signals indicating that GL-5 was terminated by Gal(β1–4)Gal. By comparing the GL-5 spectrum with the nLc4Cer and

Fig. 1. HPTLC of neutral glycosphingolipids of ostrich liver. (A) Chemical visualization with phenol/sulfuric acid; (B and C) immunostaining with human sera reactive with Galβ4Gal upon ELISA ((B) serum 97 at 1:500 dilution, and (C) serum 81 at 1:1000 dilution); (D) immunostaining with affinity-purified anti-Galβ4Gal antibodies. Immunostainings were visualized with HPR-conjugated anti-human IgM. Lane 1, neutral glycolipids of pig kidney used as standard (14 nmol); lane 2, neutral glycolipids of ostrich liver (5 nmol); lanes 3, 5, and 8, neutral glycolipids of ostrich liver (2.5 nmol); lanes 4 and 7, purified GL-5 of ostrich liver (0.1 nmol); lane 6, purified Forssman pentaglycosylceramide (F-5) of ostrich liver (0.4 nmol). Chromatography was developed in chloroform/methanol/water (60:35:8). GL-1, monohexosylceramide; GL-2, dihexosylceramide; afucoB-5, IV3-α-Gal-nLc4Cer; F-5, IV3-α-GalNAc-Gb,Cer; GL-5, IV3-β-Gal-nLc4,Cer characterized in this paper. Nomenclature of glycolipids follows the IUPAC-IUB recommendations (1997).
Galβ4Gal-terminated glycolipid

Galβ4Gal-terminated glycolipid

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signals at 4.178 and 4.706 p.p.m. were assigned to β-Glc I-1 and β-GlcNAc III-1, respectively. Signals for the three β-Gal H-1 protons were more difficult to assign. The most deshielded signal at 4.290 p.p.m. probably originated in the internal β-Gal II-1, as in IV3-α-Gal-nLc4Cer (Table I). The two other β-Gal H-1 signals were almost superimposed. The β-Gal IV-1 proton which resonates upfield when it is terminal (Table I; Levery et al., 1988) must be deshielded by the presence of β-Gal V, although to a lesser extent than by α-Gal V (Table I; Bouhours et al., 1997). In addition, according to spectra obtained in D2O of terminal Galβ4Gal oligosaccharide of Collocalia salivary mucin (Wieruszewski et al., 1987) and fish fertilized eggs glycoproteins (Taguchi et al., 1995), the terminal β-Gal H-1 proton is more deshielded than the penultimate β-Gal H-1 proton. Thus, the β-Gal proton signals at 4.273 and 4.278 p.p.m. were tentatively assigned to β-Gal IV-1 and V-1, respectively. The lactosamine core structure was supported by the β-GlcNAc NA信号 in the acetamido-methyl region at 1.835 p.p.m.

The ceramide portion of GL-5 was clearly characterized by the nonhydroxylated fatty acid signal at 2.04 p.p.m., and signals at 5.39, 5.57, and 1.96 p.p.m. (R4, R5 and R6, respectively) characteristic of unsaturated dihydroxylated sphingosine, in agreement with the findings of MS analysis.

In conclusion, the analyses fully support the structure IV4-β-Gal-nLc4Cer. It has the nLc4 core structure of blood group ABH-active and α3Gal-terminated glycolipids. As the epitope Galβ4Gal has never been found in man or in mammals, the occurrence of antibodies against this determinant was investigated.

Determination of the reactivity of human sera with the xenoepitope Galβ1–4Gal

108 human sera were tested by ELISA with Galβ4Gal-PAA as solid phase antigen. It was found that anti-Galβ4Gal antibodies do exist and that they are mainly of the IgM isotype. Anti-Galβ4Gal IgM followed the same pattern of distribution as that of natural anti-Galα3Gal and anti-Forssman IgM (Figure 5). High reactivity (absorbance over 1.0) was found in 6 sera for anti-Galβ4Gal, 11 sera for anti-Galα3Gal and 3 sera for anti-Forssman IgM (Table II). High anti-Galα3Gal reactivity was associated with the anti-Galβ4Gal reactivity in two sera, with additional anti-Forssman reactivity in one serum. It was concluded that anti-Galβ4Gal IgMs represent a new type of human natural antibodies. The reactivity of human serum IgG with Galβ4Gal-PAA was almost nonexistent (90% below
absorbance 0.25) (Figure 6). However, four sera had high IgG reactivity (absorbance over 1.0), corresponding to high anti-Gal\(^{\beta}4\)Gal IgM reactivity for three of them.

Human sera with high anti-Gal\(^{\beta}4\)Gal activity upon ELISA recognized several glycolipids in addition to IV\(^4\)-β-Gal-\(\alpha\)-Gal-\(\beta\)-Glc-\(\alpha\)-Gal-\(\beta\)-nLc4Cer. In order to know whether these glycolipids had Gal\(^{\beta}4\)Gal-terminated carbohydrate chains, anti-Gal\(^{\beta}4\)Gal antibodies were affinity-purified on a column made of the trisaccharide Gal\(^{\beta}4\)Gal\(^{\beta}4\)Glc covalently bound by a spacer arm to Sepharose. Upon ELISA, the affinity-purified anti-Gal\(^{\beta}4\)Gal IgM reacted as the total serum with the synthetic disaccharide Gal\(^{\beta}4\)Gal-PAA, and also with IV\(^4\)-α-Gal-\(\alpha\)-Gal-\(\beta\)-nLc4Cer. Furthermore, the purified anti-Gal\(^{\beta}4\)Gal IgM immunostained the same glycolipids (Figure 1D) as the whole serum (Figure 1C). Such an experiment (affinity-purification of antibodies, ELISA and TLC-immunostaining with the purified antibodies) was performed with several sera with high anti-Gal\(^{\beta}4\)Gal antibodies titer upon ELISA with Gal\(^{\beta}4\)Gal-PAA, with the same result. From these findings, it was concluded that, among the glycolipids occasionally stained by human sera (Figure 1B-C), a set of glycolipids shared the Gal\(^{\beta}4\)Gal epitope recognized by human natural antibodies (Figure 1D).

**Discussion**

The novel glycolipid characterized in ostrich liver is the fourth galactose-terminated pentaglycosylceramide of the neolactoseries (Table III). Glycolipid 1 (IV\(^3\)-α-Gal-nLc4Cer) was first characterized in rabbit erythrocyte membrane (Stellner et al., 1973). Subsequently, it appeared that the Galα3Gal determi-
Furthermore, synthesis in vitro of Galβ4Gal-terminated glycolipids was previously described (François-Gerard et al., 1980), whereas in pigeons and doves, it is present in mucin glycans (François-Gerard et al., 1980). Anti-P1 natural antibodies found in P2 individuals are usually of low strength. However, in case of immunological stimulation such as in pigeon breeders, their titer and strength rise considerably (Radermecker et al., 1975).

The discovery of glycolipid 4 (IV3-β-Gal-nLc4Cer) was significant only for IgM, and its distribution in a random population was similar to that of anti-Galβ3Gal and anti-Forssman IgM. No class switch was observed even in sera with high IgM reactivity, which is the fate of antibodies raised by oligosaccharides in a T cell independent manner. These findings were consistent with a natural antibody response. The study of the reactivity of affinity-purified anti-Galβ3Gal natural antibodies with ostrich liver glycolipids indicated that IV3-β-Gal-nLc4Cer shared the xenoepitope Galβ3Gal with several longer carbohydrate chain glycolipids.

Although the Galβ4Gal determinant has not been shown previously in glycolipids, it has been already described in a bird sialylated salivary mucin (Wieruszeski et al., 1987), and in N-linked penta-antennary glycan chains of a sialoglycopeptide of fish fertilized eggs (Taguchi et al., 1995). The enzyme responsible for the synthesis of the Galβ4Gal determinant is a β4-galactosyltransferase (β4Gal-T) which must operate downstream from the known β4Gal-T. It is provisionally termed Gal:β4Gal-T, in order to distinguish it from the “mammalian” β4-galactosyltransferases which catalyze the synthesis of lactose (Galβ4Glc) and/or N-acetyllactosamine (Galβ4GlcNac), and should be termed Glc(NAc):β4Gal-T.

Seven members of the human β4Gal-T gene family have been characterized (Almeida et al., 1999). As galactose and glucose

Table II. IgM reactivity with Galβ3Gal and Forssman antigen of human sera with the highest IgM reactivity with Galβ4Gal

<table>
<thead>
<tr>
<th>Serum</th>
<th>Galβ4Gal</th>
<th>Galβ3Gal</th>
<th>F-5</th>
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<tr>
<td>31</td>
<td>1.3</td>
<td>1.4</td>
<td>Below detection</td>
</tr>
<tr>
<td>42</td>
<td>1.2</td>
<td>1.7</td>
<td>0.5</td>
</tr>
<tr>
<td>64</td>
<td>1.5</td>
<td>0.7</td>
<td>0.4</td>
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<tr>
<td>81</td>
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<td>93</td>
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<td>0.7</td>
<td>0.2</td>
</tr>
<tr>
<td>97</td>
<td>1.5</td>
<td>1.2</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Results are the mean absorbance at 405 nm of triplicate ELISA for each serum.

Fig. 5. Distribution of the IgM reactivities in 108 human sera. Sera were grouped according to the range of their O.D. responses in ELISA with Galβ4Gal-PAA, Galβ3Gal-PAA and Forssman-5 glycolipid as solid phase antigens.
are epimeric at C-4. Galβ4Gal-T and Glc(NAc)β4Gal-T might be as different as Glc(NAc)β4Gal-T and β3Gal-T which are encoded by unrelated gene families (Almeida et al., 1998).

Expression of Galβ4Gal in glycolipids of different birds such as chicken (not shown) and ratites (ostrich and emu), and in glycoproteins of birds (Wieruszeski et al., 1987) and fishes (Taguchi et al., 1995) indicates that the Galβ4Gal-T has a large species distribution. It might be encoded by a gene which has been inactivated in Mammalia at an unknown time during evolution, in a similar way as the inactivation of the α3Gal-T gene in catarrhines (Galili and Swanson, 1991).

Materials and methods

Human sera

The human sera used in the present work for chromatography immunostaining and ELISA screening were random and anonymous sera taken at the central biochemistry laboratory of the hospital. They were heated at 56°C for 30 min for complement inactivation.

Purification of glycosphingolipids

Ostrich organs were collected after exsanguination, cut into small pieces and lyophilized. Lipids were extracted from lyophilized tissues by successive incubations at 70°C in methanol and chloroform/methanol (1:2). Glycolipids were purified from lipid extracts and separated into neutral and acid glycolipids as already described (Bouhours et al., 1992).

Thin layer chromatography

The glycosphingolipid composition of ostrich organs was analyzed by thin layer chromatography. The glycolipid solutions in glycoconjugated plates (Merck) were developed in chloroform/methanol/water (60:35:8). For chemical visualization, the plates were developed in ultraviolet light after spraying a 0.05% solution of primulin in acetone/water (4:1). Individualized glycolipids were scraped off the plate, extracted in chloroform/methanol/water (30:60:8), and taken up in chloroform/methanol (2:1).

Quantitative measurements

Quantities of glycolipids were determined by measurement of the sphingosine content of the solutions of glycolipids purified from the tissues, according to a described procedure, taking into consideration that there is 1 mol of sphingosine per mol of glycolipid (Bouhours and Glickman, 1976). The percentage distribution of the glycolipids of a tissue was determined by sphingosine measurement in the suspensions of silica gel containing individual glycolipids scraped off the chromatogram of 40 nmol of glycolipid solution on a 2 cm streak, after visualization with primuline.

Electrospray/ionization-ion trap mass spectrometry

Purified GL-5 was analyzed by mass spectrometry on a HP-Bruker-ESQUIRE-LC mass spectrometer (Bruker Daltonik, Bremen, Germany), following a described procedure (Bouhours et al., 1999). Ions obtained by electrospray-ion trap MS analysis of native glycolipids in the positive ion mode are sodium adducts of molecular ions [M + Na]⁺. Collision-induced dissociation of molecular ions gives rise to ions resulting from the cleavage of the glycosidic bonds. According to the nomenclature established by Domon and Costello (1988), fragments containing the non-reducing end of the oligosaccharide chain are labeled Ai, Bi, Ci, and the complementary fragments containing the aglycone Xj, Yj, Zj (Figure 2, MS²). The major ions observed after dissociation of the molecular ion in the ion trap were Yj = [Yj+H+Na]⁺; Bi = [B-H+Na]⁺; Ci = [C+H+Na]⁺ and Zj = [Zj-H+Na]⁻.

Methylation analysis

The carbohydrate composition and sugar linkages were determined by gas chromatography of the partially methylated alditol acetates. The purified glycolipid was permethylated by the method of Ciucanu and Kerek (1984), then submitted to acetylation, reduction with potassium borohydride and acetylation (Yang and Hakomori, 1971). Additional data on sugar sequence were obtained by reduction of the permethylated glycolipid before acetylation, using LiAIH₄ in diethyl ether according to Karlsson (1974). Gas chromatography of the partially methylated alditol acetates was done on a Hewlett Packard 5890 gas chromatograph as already described (Bouhours et al., 1997).

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### Table III. Galactose-terminated neolactoseries pentaglycosyleramides

<table>
<thead>
<tr>
<th>Structure</th>
<th>Linear formation</th>
<th>Reference</th>
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<td>1</td>
<td>Galα3Galβ3GlcNAcβ3Galβ4GlcCer</td>
<td>Stellner et al., 1973</td>
</tr>
<tr>
<td>2</td>
<td>Galβ3Galβ4GlcNAcβ3Galβ4GlcCer</td>
<td>Stellner and Hakomori, 1974</td>
</tr>
<tr>
<td>3</td>
<td>Galα3Galβ4GlcNAcβ3Galβ4GlcCer</td>
<td>Naiki et al., 1975</td>
</tr>
<tr>
<td>4</td>
<td>Galβ4Galβ4GlcNAcβ3Galβ4GlcCer</td>
<td>This work</td>
</tr>
</tbody>
</table>

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1H-NMR spectroscopy

Data on the anomerity of the sugar linkages and on the degree of hydroxylation of the fatty acids and sphingoid base were obtained by 1H-NMR spectroscopy. The native glycolipid was equilibrated three times in deuterated methanol, and dried under nitrogen. The deuterated glycolipid was dissolved in 0.5 ml of Me2SO-d6/2%H2O. Spectra were recorded at 400 MHz with 0.4 Hz digital resolution on a Bruker ARX-400 spectrometer. Chemical shifts are given relative to tetramethylsilane.

Immunoreactivity of human serum

The screening of human sera for their level of xenoreactivity was performed by ELISA according to a published technique (Rieben et al., 1995) with some modifications. The antibodies were polyacrylamide-linked disaccharides Gal(1→4)Gal-PAA and Gal(α1→3)Gal-PAA (Syntese, Munich, Germany) (Bovin, 1998). They were coated on Nunc Maxisorp microtiter plate (0.5 µg of disaccharide in 50 µl of PBS, pH 7.4). Purified Galα3-, Galβ4- and Forssman pentaglycosylceramides were coated on Dynex Immulon-1B plate (25–50 pmol in 50 µl of methanol/water (80:20) per well). Coating was performed at 4°C overnight for the PAA-disaccharides, and at room temperature for 1 h for the glycolipids. For blocking the non-specific interactions, the plates were washed three times and let to stand to 30 min at room temperature with 50 µl of PBS pH 7.4 containing 1% BSA and 1% Tween 20 (PBS/BSA/Tween). Sera were diluted 1:40 in PBS/BSA/Tween, and the reaction was performed with 50 µl of diluted serum. A blank was done for each serum, using PBS or methanol/water instead of the coating solution. After 2 h at 37°C, plates were washed five times with 50 µl of PBS containing 0.05% Tween 20 (PBS/Tween), and then incubated 1 h at 37°C with HRP-conjugated anti-human IgM (Dako) diluted 1:1000 in PBS/Tween. Enzyme activity was determined with ABTS (Boehringer) as substrate. Color intensity was measured as O.D. at 405 nm in a Dynatech MRX microplate reader after incubation for 1 h at 37°C.

Affinity-purification of anti-Galβ4Gal antibodies

Human serum (1 ml) was chromatographed on a column made with 2 ml of Galβ4Galβ4Glc-spacer-Sepharose (Syntese) in PBS. The retained antibodies were eluted with 1% ammonium hydroxide. Eluates were immediately neutralized with 1 M KH2PO4. Affinity-purified immunoglobulins were used at the same dilution as the initial serum for TLC-immunostaining and ELISA.

Acknowledgments

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Abbreviations

GL, glycosphingolipids; F, Forssman glycolipid; ESI-IT-MS, electrospray/ionization-ion trap mass spectrometry; GC, gas chromatography; HPTLC, high performance thin layer chromatography; Me2SO-d6, hexadeuterated dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay.

References


