Glycoconjugate Journal (1997) **14:** 241–248

Neutral glycosphingolipids and gangliosides from spleen T lymphoblasts of genetically different inbred mouse strains

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The gangliosides G_{M1b}, GalNAc-G_{M1b} and $G_{D1\alpha}$ are typical compounds of concanavalin A stimulated splenic T lymphoblasts of CBA/J inbred mice. Their structural characterization has been described in previous studies. The intention of this work was the comparative TLC immunostaining analysis of the glycosphingolipid composition of lectin stimulated splenic T lymphoblasts obtained from six genetically different inbred mouse strains. The strains examined were AKR, BALB/c, C57BL/6, CBA/J, DBA/2 and WHT/Ht, which are commonly used for biochemical and immunological studies. The neutral glycosphingolipid $GgOse_4Cer$, the precursor for G_{M1b}-type gangliosides, was expressed by all six strains investigated. AKR, C57BL/6 and DBA/2 showed high and BALB/c, CBA/J and WHT/Ht diminished expression in T lymphoblasts, based on single cell calculation. The gangliosides G_{M1b} and GalNAc-G_{M1b}, elongation products of $GgOse_4Cer$, displayed strain-specific differences in their intensities, which were found to correlate with the intensities of $GgOse_4Cer$ expression of the same strains. Concerning sialic acid substitution of gangliosides, G_{M1b} and GalNAc-G_{M1b} predominantly carry N-acetyl-neuraminic acid, whereas choleragenoid receptors G_{M1a} and Gal-GalNAc-G_{M1b}, which are also expressed by all six strains, are characterized by dominance of *N*-glycolylneuraminic acid. Two highly polar gangliosides, designated with X and Y, which have not been previously recognized in murine lymphoid tissue, were detected by positive anti-GalNAc-G_{M1b} antibody and choleragenoid binding, respectively. Both gangliosides were restricted to AKR, DBA/2 and C57BL/6 mice. The other three strains BALB/c, CBA/J and WHT/Ht are lacking these structures. In summary, the G_{M1b}-type pathway is quite active in all six strains analysed in this study. Strain-specific genetic variations in T lymphoblast gangliosides were observed with the occurrence of gangliosides X and Y. This study and data from other groups strongly indicate for G_{M1b}-type gangliosides a functional association with T cell activation and leukocyte mediated reactions.

Keywords: **GalNAc-G_{M1b}, $G_{D1\alpha}$, G_{M1b}-pathway, TLC immunostaining, T lymphocytes**

Abbreviations: **ConA, concanavalin A; GSL(s), glycosphingolipid(s); HPTLC, high-performance thin-layer chromatography; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid. The designation of the following glycosphingolipids follows the IUPAC-IUB recommendations (1977) [48] and the ganglioside nomenclature system of Svennerholm [49] for G_{M1a}-type gangliosides. Glucosylceramide or GlcCer, Glcβ1-1Cer; lactosylceramide or LacCer, Galβ1-4Glcβ1-1Cer; gangliotriaosylceramide or $GgOse_3Cer$ or Gg3, GalNAcβ1-4Galβ1-4Glcβ1-1Cer; gangliotetraosylceramide or $GgOse_4Cer$ or Gg4, Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer; gangliopentaosylceramide or $GgOse_5Cer$, GalNAcβ1-4Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer; gangliohexaosylceramide or $GgOse_6Cer$, Galβ1-3GalNAcβ1-4Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer. G_{M3}, II^3NeuAc-LacCer; G_{M1} or G_{M1a}, II^3NeuAc-$GgOse_4Cer$; G_{M1b}, IV^3NeuAc-$GgOse_4Cer$; GalNAc-G_{M1b}, IV^3NeuAc-$GgOse_5Cer$; G_{D1a}, IV^3NeuAc, II^3NeuAc-$GgOse_4Cer$; G_{D1b}, $II^3(NeuAc)_2$-$GgOse_4Cer$; G_{D1c}, $IV^3(NeuAc)_2$-$GgOse_4Cer$; $G_{D1\alpha}$, IV^3NeuAc, III^6NeuAc-$GgOse_4Cer$. Only NeuAc-substituted gangliosides are presented in this list of abbreviations**

Introduction

Glycosphingolipids (GSLs) are amphipathic molecules which are anchored by their ceramide portions in the outer part of the lipid bilayer, primarily of the plasma membrane, their oligosaccharide chains being exposed to the extracellular space [1]. Gangliosides are characterized by the presence of one or more sialic acid units in the oligosaccharide portion. The parent compounds are *N*-acetylneuraminic acid (NeuAc) and *N*-glycolylneuraminic acid (NeuGc), which play crucial roles in various biological functions [2]. Structures, functions and metabolism of GSLs have been widely reviewed [3–8]. The immunomodulatory potential of gangliosides is well known [9–11] and from this viewpoint, a number of analyses of GSLs from immune cells have been performed, especially with mice as a model system [12–16]. Studies of the phenotypic expression of GSL cell surface markers on lymphocytes have been shown to be

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a valuable tool for subdividing different lymphocyte subpopulations [17–19] and led to the definition of functionally distinct T helper cell subsets [20, 21].

Gangliosides of the G_{M1b}-type, *ie* G_{M1b}, GalNAc-G_{M1b} and $G_{D1\alpha}$, are characteristic GSLs of mitogen stimulated murine splenic T lymphocytes [14, 18, 22], and the Gal-NAc-G_{M1b} biosynthetic elongation product Gal-GalNAc-G_{M1b} has been isolated from mouse spleens [23]. To analyse complex GSL patterns, especially if only small amounts are available, the TLC immunostaining technique has recently proved to be a powerful tool for determination of qualitative as well as quantitative differences of the GSL pattern in various cells and tissues [24]. The aim of this work was a comparative TLC immunostaining analysis of the GSL composition of lectin stimulated splenic T lymphocytes from six genetically different inbred mouse strains, which are commonly used for biochemical and immunological studies. In general they all express the above mentioned G_{M1b}-derived gangliosides, but with a considerable quantitative diversity. In addition, three of the six strains exhibited qualitative differences due to the expression of two highly polar gangliosides (longer than Gal-GalNAc-G_{M1b}) which have never been detected before in murine leukocytes.

Materials and methods

Animals

Female CBA/J and AKR inbred mice 6–8 weeks of age were purchased from Gl Bomholtgard Ltd (Ry, Denmark) and female DBA/2, C57BL/6, and BALB/c mice from the Zentralinstitut für Versuchstierzucht (Hannover, Germany). A breeding nucleus of WHT/Ht mice was a generous gift from P. L. Russel (Grey Laboratory, Northwood, Middlesex, UK).

Murine spleen T lymphoblasts, cell culture, and mitogen stimulation

Spleen lymphocytes were isolated and cultivated as described previously [18]. They were stimulated for 48 h with 2 $\mu g\,ml^{-1}$ of the mitogen concanavalin A (ConA, Pharmacia Fine Chemicals, Freiburg, Germany). Resulting T blasts were separated from nonproliferating and dead cells by centrifugation on an one-step gradient on Lymphoprep (Nyegard, Oslo; 1.077 $g\,ml^{-1}$) and then further cultivated in the presence of the T cell growth factor interleukin 2 (IL2) as previously described [18]. T lymphoblasts were seeded with 5×10^5 and harvested at 2.5×10^6 cells ml^{-1}. After centrifugation, the sediments were suspended in chloroform/methanol (2:1 v/v) and stored at $-20°$ C.

Metabolic labelling of T lymphoblasts

T blasts propagated with IL2 (see above) were labelled for 60 h with 1 $\mu Ci\,ml^{-1}$ (37 $kBq\,ml^{-1}$) D-[1-^{14}C]glucosamine hydrochloride (54 $mCi\,mmol^{-1}$ or 2 $GBq\,mmol^{-1}$) and 1 $\mu Ci\,ml^{-1}$ (37 $kBq\,ml^{-1}$) D-[1-^{14}C]galactose (57 $mCi\,mmol^{-1}$ or 2.11 $GBq\,mmol^{-1}$; Amersham Buchler, Braunchweig, Germany). Labelled cells were harvested by centrifugation, washed twice with 0.85% NaCl supplemented with 1 mM galactose and 1 mM glucosamine, suspended in chloroform/methanol (2/1 v/v), and stored at $-20°$ C.

Isolation of GSLs

The isolation of GSLs was performed by successive extraction of the cell sediment with chloroform/methanol (2:1, 1:1, 1:2 each by vol). After evaporation the extract was dissolved in chloroform/methanol/water (30:60:8 v/v/v). Neutral GSLs and gangliosides were separated by anion exchange chromatography [14]. Gangliosides were eluted with chloroform/methanol/0.8 M sodium acetate (30:60:8 v/v/v) and desalted by use of Sep-Pak C_{18} cartridges (Millipore, Milford, MA) as described by Williams and McCluer [25]. Final purification was performed on a small Iatrobeads column (6RS-8060; Macherey-Nagel, Düren, Germany) according to Ueno *et al.* [26]. Neutral GSLs were further purified by silica gel 60 chromatography and recovered in the chloroform/methanol (40:60 v/v) eluate. Radioactivity was determined in a Tri-Carb 1900 CA liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, IL) and presented as counts per min (cpm).

Thin-layer chromatography

Glycosphingolipids were separated on high-performance thin-layer chromatography plates (HPTLC plates, size 10 cm × 10 cm, thickness 0.2 mm, E. Merck, Darmstadt, Germany). Neutral GSLs and gangliosides were separated by two solvent systems, respectively: (1) chloroform/methanol/water (120:70:17); (2) chloroform/methanol/water (120:85:20) containing 2 mM $CaCl_2$ (all ratios are v/v). Radioactive GSLs were located by autoradiography on Hyperfilm-^{3}H (Amersham Buchler).

TLC immunostaining

The detailed procedure was carried out according to Bethke *et al.* [27] and modified by the use of glycine buffer (pH 10.4) as solution for the dye [18]. Monoclonal mouse IgM anti-$GgOse_3Cer$ antibody [28] from the hybridoma clone 2D4 (TIB 185), obtained from the American Type Culture Collection (ATCC, Bethesda, MD), was used as a 1:10 dilution of culture supernatant. Polyclonal rabbit anti-$GgOse_4Cer$ antibody was prepared according to the method of Kasai *et al.* [29] and used as a 1:1000 dilution [27]. Secondary goat anti-mouse IgM and anti-rabbit IgG antibodies, both labelled with alkaline phosphatase, were purchased from Dianova (Hamburg, Germany) and diluted 1:2000 for TLC immunostaining. 5-bromo-4-chloro-3-indolylphosphate was used as enzyme substrate.

TLC immunostain of G_{M1b} *type gangliosides*

Terminally sialylated gangliosides with $GgOse_4Cer$ backbone (G_{M1b}, $G_{D1\alpha}$) were detected as described previously

[30]. After chromatography of gangliosides and silica gel fixation, the plate was incubated for 2 h at room temperature with 5 mU ml^{-1} *V. cholerae* neuraminidase (Behring Werke AG, Marburg, Germany). Desialylated gangliosides were immunostained with polyclonal anti-$GgOse_4Cer$ antibody (see above).

The overlay assay for detection of GalNAc-G_{M1b} using a highly specific chicken antiserum has been described in detail elsewhere [31, 32]. Alkaline phosphatase labelled second anti-chicken IgG antibody was obtained from Dianova and diluted 1 : 1000.

TLC immunostain of G_{M1a} type gangliosides and Gal-GalNAc-G_{M1b}

The TLC binding method with cholera toxin B subunit (choleragenoid) for specific detection of G_{M1a} has been developed by Magnani *et al.* [33] and was used according to the modifications described by Pörtner *et al.* [26]. For the identification of G_{M1a} and Gal-GalNAc-G_{M1b} the TLC-plate was incubated for 2 h at room temperature with 250 ng ml^{-1} choleragenoid (Sigma, Deisenhofen, Germany; no. C-7771) followed by overlaying with goat anti-choleragenoid antiserum (Calbiochem, Frankfurt a. M., Germany; no. 227040) and alkaline phosphatase conjugated rabbit anti-goat IgG antibody (Dianova), both diluted 1 : 1000.

Quantitative determination of TLC immunostained GSLs

For quantitative estimation immunostained chromatograms were scanned with a CD60 scanner (Desaga, Heidelberg, Germany) equipped with an IBM compatible personal computer and densitometric software. The bands were measured in reflectance mode at 630 nm with a light beam slit of 0.1 mm × 2 mm [24]. Overall peak areas of each GSL obtained from all strains were set to 100%, and relative amounts for the immunostained GSLs of each strain were calculated on a percentage basis.

Results

Neutral GSLs and gangliosides from ConA stimulated splenic T lymphocytes (= T lymphoblasts) of the inbred CBA/J mouse strain have been structurally characterized in earlier reports. Structures of gangliosides of the G_{M1a}- and the G_{M1b}-pathway are depicted as a diagram in Figure 1. Main monosialogangliosides have been identified as G_{M1b} and GalNAc-G_{M1b}, substituted with C_{24}- and C_{16}-fatty acids as well as NeuAc and NeuGc deduced from mass spectrometric data [14]. Both compounds have previously been reported to be characteristic GSLs of mouse spleen [23, 34]. The mentioned heterogeneity leads to double bands on thin-layer chromatograms of *eg* G_{M1b}(NeuAc)- as well as G_{M1b}(NeuGc)-pairs due to C_{24}- and C_{16}-fatty acids (upper and lower bands, respectively) for each NeuAc- and NeuGc-derivative. By use of solvent II for TLC separation

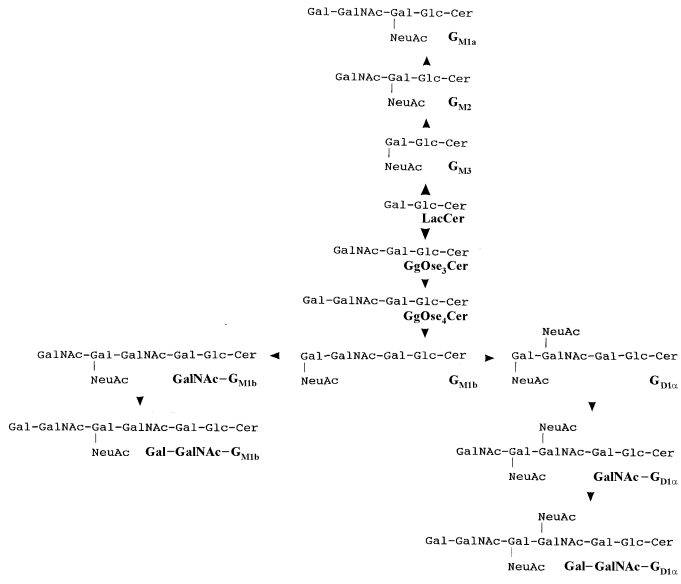

Figure 1. Structures of gangliosides of the G_{M1a}- and the G_{M1b}-pathway. Only NeuAc-substituted gangliosides are shown in this diagram.

of gangliosides, these four compounds chromatograph in three bands (see Table 1). The upper one represents G_{M1b}(NeuAc, C_{24}-fatty acid), the lower one G_{M1b}(NeuGc, C_{16}-fatty acid) and the middle band contains co-migrating gangliosides G_{M1b}(NeuAc, C_{16}-fatty acid) and G_{M1b} (NeuGc, C_{24}-fatty acid). Identical TLC behaviour was found for the four GalNAc-G_{M1b} variants [14]. YAC-1, a murine T lymphoma, was later reported to express same G_{M1b} and GalNAc-G_{M1b} gangliosides [31, 35]. The main disialogangliosides of spleen T lymphoblasts of CBA/J strain were identified as $G_{D1\alpha}$(NeuAc, NeuAc) carrying C_{24}- and C_{16}-fatty acid (upper and lower band) as previously reported [18, 30]. The aim of this work now was to elucidate whether the G_{M1b}-type pathway of ganglioside biosynthesis is restricted to the CBA/J strain, from which these structural data were obtained, or whether these GSLs are characteristic and widely distributed components of T lymphoblasts from genetically different mouse inbred strains.

Neutral GSLs of T lymphoblasts of different mouse strains

The TLC autoradiogram of ^{14}C-labelled neutral GSLs of T lymphoblasts from all six mouse strains is shown in Figure 2A. Main compounds are $GgOse_3Cer$ and $GgOse_4Cer$; the latter is the direct precursor for G_{M1b}-type gangliosides (G_{M1b}, GalNAc-G_{M1b} and $G_{D1\alpha}$). As demonstrated in Figure 2B, quantitative differences of $GgOse_4Cer$ were detected by TLC immunostain with anti-$GgOse_4Cer$ antibody desired from equal cell numbers (5×10^6 T blasts). Highest amounts were found in C57BL/6, AKR and DBA/2 mice (Figure 1B, lanes c, a and e, respectively). Considerable lower expression was detected in WHT/Ht, CBA/J and BALB/c strains (Figure 1B, lanes f, d and b, respectively). In some cases the

Table 1. Structures of G_{M1b}-type gangliosides from ConA stimulated CBA/J mouse splenic T lymphocytes.[a]

Ganglioside fraction	*Fatty acid*	*Structure*	*Symbol*
I	C_{24}	IVNeuAc-$GgOse_4Cer$	G_{M1b}(NeuAc)[b]
II	C_{16}	IVNeuAc-$GgOse_4Cer$	G_{M1b}(NeuAc)[b]
	C_{24}	IVNeuGc-$GgOse_4Cer$	G_{M1b}(NeuGc)[b]
	C_{24}	IVNeuAc-$GgOse_5Cer$	GalNAc-G_{M1b}(NeuAc)[c]
III	C_{16}	IVNeuGc-$GgOse_4Cer$	G_{M1b}(NeuGc)[b]
	C_{16}	IVNeuAc-$GgOse_5Cer$	GalNAc-G_{M1b}(NeuAc)[c]
	C_{24}	IVNeuGc-$GgOse_5Cer$	GalNAc-G_{M1b}(NeuGc)[c]
IV	C_{16}	IVNeuGc-$GgOse_5Cer$	GalNAc-G_{M1b}(NeuGc)[c]
V	C_{24}	IVNeuAc, IIINeuAc-$GgOse_4Cer$	$G_{D1\alpha}$(NeuAc,NeuAc)[b]
VI	C_{16}	IVNeuAc, IIINeuAc-$GgOse_4Cer$	$G_{D1\alpha}$(NeuAc, NeuAc)[b]

[a]Data drawn from Müthing *et al.* [14, 18]; [b]detectable by TLC immunostain with the anti-$GgOse_4Cer$ antibody after *V. cholerae* neuraminidase treatment [30]; [c]detectable by TLC immunostain with anti-GalNAc-G_{M1b} antibody [31, 32].

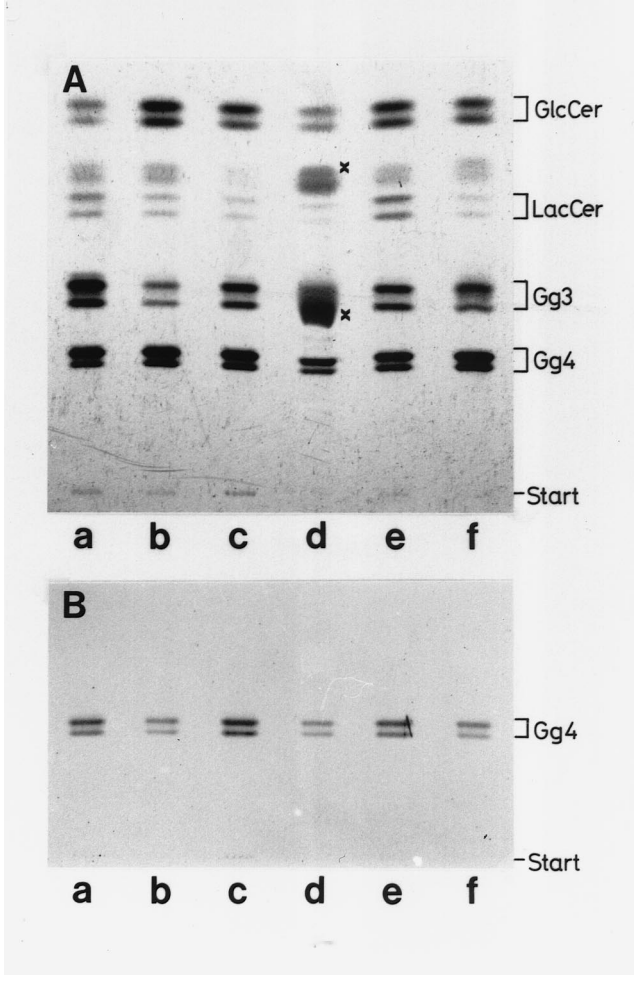

Figure 2. Autoradiography (A) and TLC immunostain with anti-$GgOse_4Cer$ antibody (B) of neutral GSLs from T lymphoblasts of different mouse strains. In (A) 5000 cpm of each ^{14}C-labelled neutral GSL fraction and in (B) neutral GSLs corresponding to 5×10^6 T blasts were applied and chromatographed in solvent I. (a) AKR, (b) BALB/c, (c) C57BL/6, (d) CBA/J, (e) DBA/2, (f) WHT/Ht. Exposure time of autoradiography was 108 h. Crosses in lane d indicate non-GSL impurities.

TLC immunostain does not correspond quantitatively to the radioactivity incorporated into $GgOse_4Cer$ (*eg* Figure 2A, B, lanes b and f). This is due to different incorporation rates of radioactive monosaccharide precursors supplied in the growth medium [14], whereas the TLC immunostain reflects the real amounts of GSLs on a per cell basis. However, co-migration of $GgOse_4Cer$ with other GSLs can be excluded. A synopsis of the relative quantities of each TLC immunostained GSL of the six different inbred strains (see below) is provided in Table 2.

Gangliosides of T lymphoblasts of different mouse strains

The TLC autoradiography of ^{14}C-labelled T lymphoblast gangliosides from all mouse strains is shown in Figure 3. The pattern seemed to be very similar, however, unequal intensities of bands I to VI indicate quantitative differences. Bands I to IV comprise G_{M1b}- and GalNAc-G_{M1b}-variants, whereas bands V and VI, which chromatograph between reference G_{D1a} and G_{D1b} of human brain (see Figure 3), represent $G_{D1\alpha}$ disialogangliosides. Since it is known from former analysis of CBA/J strain that 'single' bands separated by TLC bear up to three different gangliosides of G_{M1b}-type (see Table 1), quantitative determination of G_{M1b}, GalNAc-G_{M1b} and $G_{D1\alpha}$ was performed by TLC immunostaining (see Table 2). Furthermore, the extremely sensitive combined choleragenoid-immunostaining assay on TLC plates was used for detection of G_{M1} and Gal-GalNAc-G_{M1b} as shown below.

TLC immunostaining of G_{M1b} and $G_{D1\alpha}$ in ganglioside fractions of different mouse strains

Ganglioside aliquots corresponding to 1.5×10^6 T blasts of each strain were separated and G_{M1b} and $G_{D1\alpha}$ were immunostained with anti-$GgOse_4Cer$ antibody after *V. cholerae* neuraminidase treatment (Figure 4). Control stains with anti-$GgOse_4Cer$ antibody without neuraminidase treatment were negative. All strains were found to express

Table 2. Synopsis of relative quantitation of each TLC immunostained GSL from six different inbred mouse strains.[a]

Lane[b]	*Strain*	*$GgOse_4Cer$*	*G_{M1b}*	*G_{M1a}*	*G_{D1a}*	*GalNAc-G_{M1b}*	*Gal-GalNAc-G_{M1b}*	*X*	*Y*
a	AKR	21	26	20	24	20	14	20	22
b	BALB/c	7	7	13	11	14	15	1	2
c	C57BL/6	34	22	12	19	15	11	44	36
d	CBA/J	8	11	12	15	11	18	nd[c]	nd
e	DBA/2	20	27	22	23	29	22	35	32
f	WHT/Ht	10	7	21	8	11	20	nd	8

[a]All data are expressed as percentage relative amounts; peak areas of TLC immunostain densitograms for each GSL of all six strains were set to 100%, respectively.
[b]According to all figures.
[c]nd, not detected.

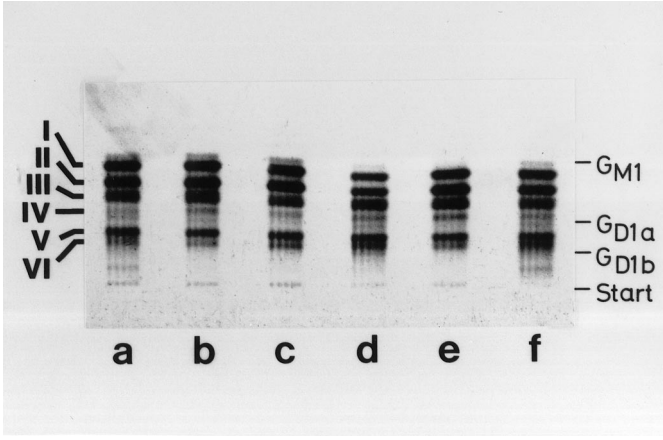

Figure 3. Autoradiography of ^{14}C-labelled gangliosides from T lymphoblasts of different mouse strains. 5000 cpm of each ganglioside fraction were applied and chromatographed in solvent II. (a) AKR, (b) BALB/c, (c) C57BL/6, (d) CBA/J, (e) DBA/2, (f) WHT/Ht. Exposure time was 108 h. Main compounds of fractions I to VI are listed in Table 1. The positions of reference gangliosides G_{M1}, G_{D1a} and G_{D1b} from human brain are marked on the right margin.

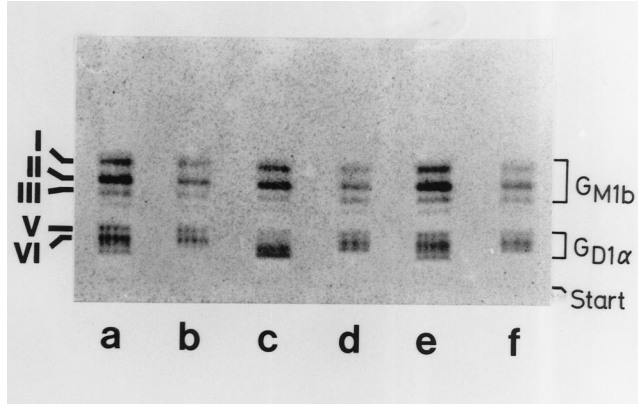

Figure 4. TLC immunodetection of G_{M1b} and $G_{D1\alpha}$ in ganglioside fractions of T lymphoblasts of different mouse strains. Gangliosides corresponding to 1.5×10^6 T blasts were applied and chromatographed in solvent II. After *V. cholerae* neuraminidase treatment, desialylated gangliosides were immunostained with anti-$GgOse_4Cer$ antibody. (a) AKR, (b) BALB/c, (c) C57BL/6, (d) CBA/J, (e) DBA/2, (f) WHT/Ht.

three G_{M1b}-bands, comprising four G_{M1b}-variants due to substitution with C_{24}- and C_{16}-fatty acid, and with NeuAc and NeuGc (see Table 1). Due to identical chromatographic behaviour of GSLs from CBA/J strain, which structures have been determined earlier by mass spectrometry analysis [14, 30], and the other five strains, substitution with C_{24}- and C_{16}-fatty acids in their ceramides is suggested. Intensities of G_{M1b}-bands differed, indicating higher quantities in DBA/2, AKR and C57BL/6 mice (Figure 4, lanes e, a and c, respectively; see Table 2) and less amounts in CBA/J, BALB/c and WHT/Ht mice (Figure 4, lanes d, b and f, respectively). However, the ratios of the single bands within each G_{M1b}-pattern were almost identical in the six strains. Compared to the pattern of precursor neutral GSL $GgOse_4Cer$ (see Figure 2), the quantitative differences among the strains were similar. In all strains prevelence of G_{M1b}(NeuAc) with C_{24}- and C_{16}-fatty acid of ganglioside fractions I and II over the NeuGc-derivatives G_{M1b}(NeuGc) with C_{24}- and C_{16}-fatty acid of ganglioside fractions II and III is obvious (see also Table 1). This phenomenon is in accordance to previous structural characterization of G_{M1b} and GalNAc-G_{M1b} of CBA/J mice, mainly substituted with NeuAc [14], and of $G_{D1\alpha}$(NeuAc, NeuAc) of this strain, even lacking NeuGc-substitution [18]. Chromatography of ganglioside fractions in alkaline solvent, which results in enhanced separation of NeuAc- and NeuGc-substituted GSLs, did not facilitate identification of these ganglioside species due to interference of NeuGc-containing mono- and NeuAc-containing disialogangliosides. Some heterogeneity among the strains was observed on the level of $G_{D1\alpha}$, which was identified on the basis of comigration with reference $G_{D1\alpha}$ from CBA/J-strain, especially by the presence of more polar $G_{D1\alpha}$-type gangliosides below band VI in case of AKR, C57BL/6 and DBA/2 strains (Figure 4, lanes a, c and e, respectively).

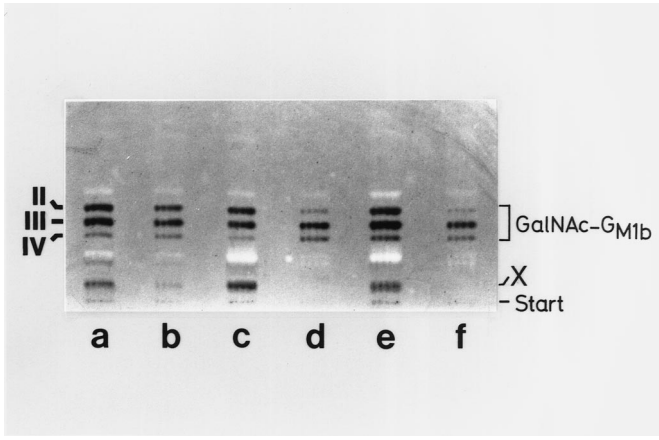

Figure 5. TLC immunostain of GalNAc-G_{M1b} in ganglioside fractions of T lymphoblasts of different mouse strains. Gangliosides corresponding to 5×10^6 T blasts were applied and chromatographed in solvent II. (a) AKR, (b) BALB/c, (c) C57BL/6, (d) CBA/J, (e) DBA/2, (f) WHT/Ht.

TLC immunostaining of GalNAc-G_{M1b} in ganglioside fractions of different mouse strains

The overlay-assay was done with specific anti-GalNAc-G_{M1b} antibody, which recognizes both, NeuAc- and NeuGc-substituted GalNAc-G_{M1b} variants [31]. Compared to G_{M1b}-expression demonstrated above (Figure 4), similar patterns of the G_{M1b}-elongation product were obtained (Figure 5), also suggesting the dominance of NeuAc-substitution of GalNAc-G_{M1b} in most of the strains. However, there appears to be more product in species III and IV, suggesting dominance of NeuGc substitution in CBA/J and WHT/Ht strain (Figure 5, lanes d and f, respectively). Again, DBA/2, AKR and C57BL/6 mice showed enhanced GalNAc-G_{M1b} expression (Figure 5, lanes e, a and c) compared to the remaining three strains (Figure 4, lanes b, d and f; see Table 2). The ones showing strongest GalNAc-G_{M1b} immunostaining expressed a single band indicating the presence of a highly polar ganglioside, designated with X in Figure 5 (lanes a, c and e). It is strongly suggested that this ganglioside represents a disialylated GalNAc-G_{M1b} derivative, presumably GalNAc-$G_{D1\alpha}$. This seems most likely because high expression of $G_{D1\alpha}$ was found in DBA/2, AKR and C57BL/6 strains (see Figure 4) and no $GgOse_6Cer$ precursor of alternative monosialoganglioside with $GgOse_7Cer$ core and terminal GalNAcβ1-4(NeuAcα2-3)Gal-sequence (to be synthesized *via* VI^3NeuAc/NeuGc-$GgOse_6Cer$ followed by elongation with GalNAc) was detected in the neutral GSL fraction (see Figure 2). Ganglioside X, detected by a highly sensitive polyclonal antibody working in the nanogram range, was not detectable by autoradiography due to its low quantity (see Figure 3). However, over-exposure of the TLC plate revealed some weak bands at the position of ganglioside X.

TLC choleragenoid-immunostaining of G_{M1} and Gal-GalNAc-G_{M1b} in ganglioside fractions of different mouse strains

As revealed from several reports, choleragenoid is known to bind to G_{M1} and Gal-GalNAc-G_{M1b} regardless of NeuAc- or NeuGc-substitution [23]. This assay is more than two orders of magnitude sensitive than antibody based TLC immunostaining assays: G_{M1} and Gal-GalNAc-G_{M1b} can be detected in picogram amounts. Thus, these compounds represent minor 'underlying' GSLs in the ganglioside fractions of mouse T lymphoblasts, which predominantly synthesize G_{M1b}-type gangliosides. To avoid confusion of the highly complex composition of monosialogangliosides of T lymphoblasts, the variants of gangliosides G_{M1a} and Gal-GalNAc-G_{M1b} reported in this chapter (see below) are not depicted in Table 1. However, their structures are shown in the diagram of Figure 1 (see above). All six strains express G_{M1} and Gal-GalNAc-G_{M1b} (Figure 6), but in distinctly different intensities (see Table 2). Contrary to G_{M1b} and GalNAc-G_{M1b} (see Figures 4 and 5), G_{M1a} (bands I and II in Figure 6) and Gal-GalNAc-G_{M1b} preferentially carry NeuGc, the upper G_{M1a}-band corresponding to G_{M1a}(NeuGc, C_{24}-fatty acid) and the lower one to G_{M1a}(NeuGc, C_{16}-fatty acid). The faint band above fraction I represents G_{M1a}(NeuAc, C_{24}-fatty acid) and underlying G_{M1a}(NeuAc, C_{16}-fatty acid) co-chromatographs with G_{M1a}(NeuGc, C_{24}-fatty acid) in band I. The same pattern of distribution is assumed for Gal-GalNAc-G_{M1b} gangliosides (Figure 6). The most interesting finding deduced from the overlay-assay in Figure 6 was the distinguishment of the strains AKR, C57BL/6 and DBA/2 by expression of a low migrating ganglioside designated with Y (Figure 6, lanes a, c and e). This ganglioside is

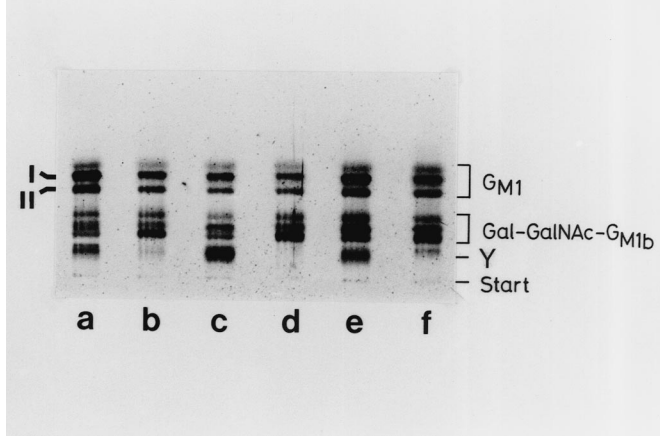

Figure 6. Combined TLC choleragenoid and immunostain of G_{M1} and Gal-GalNAc-G_{M1b} in ganglioside fractions of T lymphoblasts of different mouse strains. Gangliosides corresponding to 5×10^6 T blasts were applied and chromatographed in solvent II. The plate was overlayed with choleragenoid, followed by incubation with goat anti-choleragenoid and alkaline phosphatase conjugated rabbit anti-goat IgG antibodies. (a) AKR, (b) BALB/c, (c) C57BL/6, (d) CBA/J, (e) DBA/2, (f) WHT/Ht.

suggested to represent the disialoganglioside Gal-GalNAc-$G_{D1\alpha}$ due to arguments explained in the former chapter (see above). Due to extremely low amounts of ganglioside Y, this ganglioside is not visable on the autoradiogram (Figure 3) as explained above for ganglioside X. Chromatography of gangliosides in alkaline solvent led to interference of NeuGc-substituted G_{M1a}-variants and NeuAc-substituted Gal-GalNAc-G_{M1b} species, preventing unequivocal identification of gangliosides in this solvent.

Discussion

According to the results obtained in this study with ConA stimulated splenic T lymphocytes, the six genetically different mouse strains can be divided into two groups by their GSL expression. AKR, C57BL/6 and DBA/2 showed enhanced G_{M1b}-expression compared to BALB/c, CBA/J and WHT/Ht mice. However, all strains displayed almost identical proportions within respective G_{M1b}-patterns. Low and high expression of G_{M1b} was correlated to low and high quantities of $GgOse_4Cer$–the direct precursor of G_{M1b}–and elongated G_{M1b}-type gangliosides. $G_{D1\alpha}$ the main disialoganglioside from CBA/J splenic T blasts [18], was shown by TLC immunostaining to be present in all six strains, however, quantitative as well as qualitative differences have been observed.

The WHT/Ht mouse strain is the most distinguished due to its specific block in the biosynthesis of gangliosides longer than G_{M3} (which are G_{M2}, G_{M1} and disialogangliosides of the G_{M1a}-pathway). This phenomenon is characteristic for mouse erythrocytes and liver cells [36, 37] and this genetic polymorphism among genetically different mouse strains was found to be restricted to those two cell types, whereas other organs (including spleen and thymus) of WHT/Ht animals showed specific but nonpolymorphic patterns [38]. As demonstrated above, together with BALB/c and CBA/J animals the WHT/Ht strain falls into the category of low G_{M1b}- and $G_{D1\alpha}$-producers. All six strains shared the same pattern with the exception of the tentatively characterized gangliosides X and Y, which are, besides WHT/Ht strain, also absent in BALB/c and CBA/J mice. Thus, concerning GSL expression in WHT/Ht mice, results presented in this study are in complete agreement with data of Nakamura *et al.* [38], who showed ganglioside polymorphism to be restricted in this strain to erythrocytes and liver tissues, whereas e.g. spleen and thymus did not show any polymorphisms compared to other inbred strains.

A disialoganglioside of the G_{M1b}-pathway having the structure of $IV^3(NeuAc)_2$-$GgOse_4Cer$ (G_{D1c}) has been isolated from a spontaneous thymoma of AKR mice [39]. The same type of ganglioside, but substituted with the disialogroup α2-8NeuGc-α2-3-R at the nonreducing terminus of $GgOse_4Cer$, was found by Nakamura *et al.* [40] to be the major disialoganglioside in WHT/Ht mouse thymoma and thymocytes. Beside G_{D1c} (NeuGc, NeuGc), two more disialogangliosides of the G_{D1c}-type were tentatively characterized as G_{D1c}(NeuAc, NeuGc) and G_{D1c}(NeuGc, NeuAc) in this study, but no G_{D1c}(NeuAc, NeuAc) was detected. This GSL was recently isolated from a murine thymoma and identified as the $3G11^+$ antigen [21]. The ganglioside nature of the $3G11^+$ antigen, a marker for murine $CD4^+$ TH1 lymphocytes, has been earlier suggested by Greer *et al.* [20], and from their data the G_{D1c}-structure was obvious. In a very recent and excellent report of Nakamura *et al.* [41] the authors could demonstrate the restriction of G_{D1c}(NeuGc, NeuGc) to murine $CD4^+$ T cells and a small population of mature thymocytes. From their results obtained through cellular and physiological experiments with BALB/c, C57BL/6 and DBA/2 mice, it could be suggested that G_{D1c} represents an excellent marker for mouse naive T or T helper 1-like cells *in vivo*. Concerning the disialoganglioside expression of the six different mouse strains reported here, the question rises: Why do stimulated splenic T lymphoblasts not express G_{D1c}? The important point is, that the $3G11^+$ antigen ($= G_{D1c}$) is down-regulated after cell activation and appears to remain negative even after cells return to the resting stage [42], which gives the explanation of absence in stimulated T blasts of various mouse strains.

The G_{M1b}-elongation product GalNAc-G_{M1b}, known to be expressed in T lymphoblasts [14, 18, 22] and murine spleens [23], was present in all strains and showed distribution patterns similar to G_{M1b}. Interestingly, the tentatively identified ganglioside X (probably GalNAc-$G_{D1\alpha}$) was restricted to the high G_{M1b}-producers AKR, C57BL/6 and DBA/2. The same distribution pattern was observed for the second tentatively identified ganglioside Y (probably Gal-GalNAc-$G_{D1\alpha}$), not found in BALB/c, CBA/J and WHT/Ht mice. It should be mentioned that the choleragenoid-TLC immunostaining procedure detects gangliosides of two completely different biosynthetic pathways, i.e G_{M1} of the G_{M1a}-pathway and Gal-GalNAc-G_{M1b} of the G_{M1b}-pathway, due to homology in their carbohydrate terminus. The attempt to identify G_{D1a}, G_{D1b} etc. by choleragenoid-TLC immunostaining after *V. cholerae* neuraminidase treatment failed due to interference with Gal-GalNAc-G_{M1b}-structures on the TLC plate, preventing unambiguous identification of e.g. G_{D1a} and/or G_{D1b}.

With emphasis to functional involvement in leukocyte mediated processes, the neutral GSL $GgOse_4Cer$ has been most thoroughly examined among all GSLs. Preferential reaction of anti-$GgOse_4Cer$ antibodies towards mature T cells has been earlier investigated by Stein *et al.* [43] for several mouse inbred strains. The expression of $GgOse_4Cer$ in normal thymocyte and spleen cell populations can be induced by stimulation with mitogens reported by Suttles *et al.* [44] and Stout *et al.* [45]. Also differential expression of $GgOse_4Cer$ on alloreactive cytotoxic T lymphocytes and lymphokine-activated killer cells has been observed [46]. Within the sialylated GSLs, ganglioside G_{M1a} has recently been proposed as marker for immature T cells [47], whereas Gal-GalNAc-G_{M1b} is probably a characteristic ganglioside

of mature T cells, thus both are discussed to be markers for unique T cell populations in mice. In summary the presumed GSL markers of immature and/or mature thymocytes or T lymphocytes were found in all of the six mouse strains analysed. Not even one strain lacked any of these structures. They all shared gangliosides of the dominant G_{M1b}-pathway, starting with $GgOse_4Cer$ and resulting in G_{M1b} and elongated GalNAc-G_{M1b} and Gal-GalNAc-G_{M1b} and disialylated $G_{D1\alpha}$. The comparison of gangliosides from unstimulated thymocytes and ConA stimulated splenocytes of the CBA/J strain [18] suggested elongated G_{M1b}-type gangliosides to represent markers of mature T cells due to enhanced expression. Whether these structures can be assigned as markers for all six strains cannot be decided since only stimulated splenocytes were analysed in this study, lacking comparative stains for quiescent T cells.

Two until yet unknown gangliosides were tentatively identified by TLC immunostain to be most likely GalNAc-$G_{D1\alpha}$ and Gal-GalNAc-$G_{D1\alpha}$. Thus, further investigations are necessary to assign functional attributes to these structures in leukocyte mediated interactions.

Acknowledgements

I would like to thank Dr H. Ziehr (GBF, Braunschweig, Germany) for critical reading of the manuscript.

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Received 22 May 1996, revised 1 August 1996, accepted 1 August 1996