GLYCOLIPIDS AS MARKERS OF MURINE T AND B LYMPHOBLASTOID TUMOUR CELL LINES

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1. Introduction

Cell surface glycosphingolipids are thought to be implicated in processes of cell–cell recognition and growth regulation, receptor function, and malignant transformation [1–3]. The identification and the structural analysis of these membrane components are also expected to afford an insight into the process of thymus-derived (T) and bone marrow-derived (B) lymphocyte differentiation, since activated T and B lymphocytes express different sets of glycosphingolipids [4,5]. It is, therefore, not unreasonable to assume that tumour cell lines of T and of B cell origin will each yield characteristic glycosphingolipid distribution patterns relating either to the expression of tumour-associated moieties or to components typical for certain functional states during differentiation and/or maturation.

Here, spontaneous or chemically induced, transplantable murine T and B lymphoblastoid tumours, adapted to in vitro conditions of growth [6,7] were used in a comparative qualitative and quantitative study of ganglioside and neutral glycolipid distribution patterns revealed by high-performance thin-layer chromatography (HPTLC) after biosynthetic sugar labelling [5]. We document here differences not only between T and B cell lines but also among T and B cell lines that may be used as biochemical markers.

2. Materials and methods

The lymphoblastoid tumour cell lines used were 8-azaguanine-resistant myeloma and B cell hybridoma lines, originally derived from Balb/c myeloma MOPC 21, several AKR thymomas, one C57BL/6 thymoma, and two Fcγ-receptor bearing T cell hybridomas (table 1). These cell lines were cultivated in quadruplicates for 72 h in Dulbecco's modified Eagle's medium, containing 15% foetal calf serum, using COSTAR, no. 3524 (Cambridge, MA), tissue culture plates; only Spl/HK was grown in the presence of 15% horse serum. Initial cell concentrations were 5×10^5 cells/ml. Both 0.5 μCi/ml D-[1-^{14}C]-galactose (spec. act. 53.3 Ci/mol) and 0.5 μCi/ml D-[1-^{14}C]glucosamine hydrochloride (spec. act. 55.4 Ci/mol), both from New England Nuclear, were present for the 72 h cultivation period. Cells were harvested by centrifugation (300 × *g*) in glass tubes, washed twice with phosphate-buffered saline, and they were repeatedly extracted with chloroform: methanol (2:1 and 1:1, v/v) [5,14]. Contaminating free radioactive sugars were removed by apartition procedure as in [15]. Lipid-bound radioactivity was counted in Aquasol scintillator using an Intertechnique liquid scintillation counter (Model SL4000, Nucletron SA, Lausanne).

To achieve a more precise qualitative and quantitative determination of the material that had been biosynthetically labelled by this procedure, extracted lipids from ~10^3 cells grown in 100 ml COSTAR no. 3150 culture flasks were partitioned by a modified Folch procedure [16]. Upper phases, containing mainly gangliosides, were extensively dialysed against cold distilled water. The lower phases, comprising mainly phospholipids and neutral glycolipids, were acetylated and subsequently fractionated by Florisil column chromatography according to [16]. The neutral glycolipids obtained by this procedure and the gangliosides from the aqueous phase were then

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analysed separately on Merck silica HPTLC plates on the basis of polarity. Bands of glycolipids, that were labelled by [^{14}C]galactose and [^{14}C]glucosamine, were developed by autoradiography on Kodak RP. Royal X-Omat X-ray films. For comparison CBA/J mouse brain gangliosides, and globotriaosylceramide of human erythrocytes as well as gangliotetraosylceramide of acid-treated human brain gangliosides were applied as cold references, and they were stained with resorcinol and orcinol, respectively [14].

3. Results

Incorporation rates of ^{14}C-labelled sugars varied with different cell lines. This property correlates with the observed growth rates: e.g., clone E-7 has a considerably lower doubling time and a lower rate of incorporation of ^{14}C-labelled sugars than its parent line T2D4 (table 1). Ganglioside (fig.1) and neutral-glycolipid (fig.2) patterns of Con A and LPS stimulated spleen cell cultures yielded the established reference patterns for T and B lymphocyte stimulation [5]. These patterns were obtained with all of 10 inbred-mouse strains tested so far (G. R., unpublished).

Thymoma-derived ganglioside and neutral glycolipid distribution patterns are altogether distinct from myeloma-associated patterns. The two cell types show the greatest differences at the level of labelled gangliosides (fig.1). Myeloma lines X63 and X63-Ag8.653, and B cell hybridoma lines Sp2/0 and Spl/HK incorporate 80–90% of the ganglioside-associated radioactivity into a unique and closely circumscribed cluster near the GM3 range (table 2); this group of gangliosides accounts for only 5.5% of the LPS-stimulated B-cell patterns (table 2). It is interesting that hybridoma Sp2/0, a non-immunoglobulin secreting line [10], consistently shows an inversion in strength of band R_F 1.53 over band R_F 1.45 with a somewhat reduced band R_F 1.36 in comparison with X63 (table 2). Gangliosides of the haematoside series (GM3) separate in this chromatographic system according to the nature of their sialic acids, but also according to the polarity of their ceramides [17]. The neutral glycolipid patterns of the X63 myeloma and its derivative lines X63-Ag8.653, Spl/HK, and Sp2/0 show considerable overlaps with the LPS-stimulated B-cell associated patterns (fig.2). However, in contrast to those, tetraosylceramides are minor components. Double bands in this system represent glycolipids with common sugar moieties, but different ceramides (table 3; G. R., unpublished). Thus, the differences are likely to relate to distinct activities of the enzymes involved in the biosynthesis of ceramides.

Ganglioside patterns of thymomas and T-cell hybridomas are highly variable (fig.1, table 2).

Table 1
Incorporation efficiency of ^{14}C-labelled galactose and glucosamine into lipids of 10^7 cells

Cell line[a]	Hybridoma line[a]	Mouse strain	Cell-surface markers	cpm
1. P3/X63 Ag8 (X63)	–	BALB/c	H-2^d	24 888 ± 5782
2. X63-Ag8.653	–	BALB/c	H-2^d	14 951 ± 569
3. –	Spl/HK	BALB/c	H-2^d	13 652 ± 708
4. –	Sp2/0-Ag14 (Sp2/0)	BALB/c	H-2^d	12 405 ± 860
1. BW5147	–	AKR	H-2^k; Thy-1.1	5671 ± 1137
2. 344TC11	–	AKR	H-2^k; Thy-1.1	8823 ± 1572
3. 369 TC	–	AKR	H-2^k; Thy-1.1	4378 ± 2605
4. 424A11	–	AKR	H-2^k; Thy-1.1	33 390 ± 2957
5. EL-4 Bu(EL-4)[8]	–	C57BL/6	H-2^b	23 399 ± 2788
6. –	T2D4	AKR × B10.BR	H-2^k; Thy-1.1, Thy-1.2	21 822 ± 2234
7. –	E-7	AKR × B10.BR	H-2^k; Thy-1.1, Thy-1.2	6139 ± 1536

The cell and hybridoma lines X63 [8], Spl/HK [9], Sp2/0 [10] and BW5147 [11] were obtained from Dr G. Köhler, Basle Institute for Immunology; X-63Ag8.653 [12] was from Dr T. Imanishi-Kari, Institut für Genetik, Cologne; 344TC11, 369TC, and 42411 thymomas were from Dr P. Krammer, Deutsches Krebsforschungs Zentrum, Heidelberg; T2D4 [13] hybridoma (AKR thymoma BW5147 × B10.BR in vivo alloantigen-activated T cells) and clone E-7 [13] derived from it were from Dr W. H. Fridmann, Institut de Recherches Scientifiques sur le Cancer, Villejuif

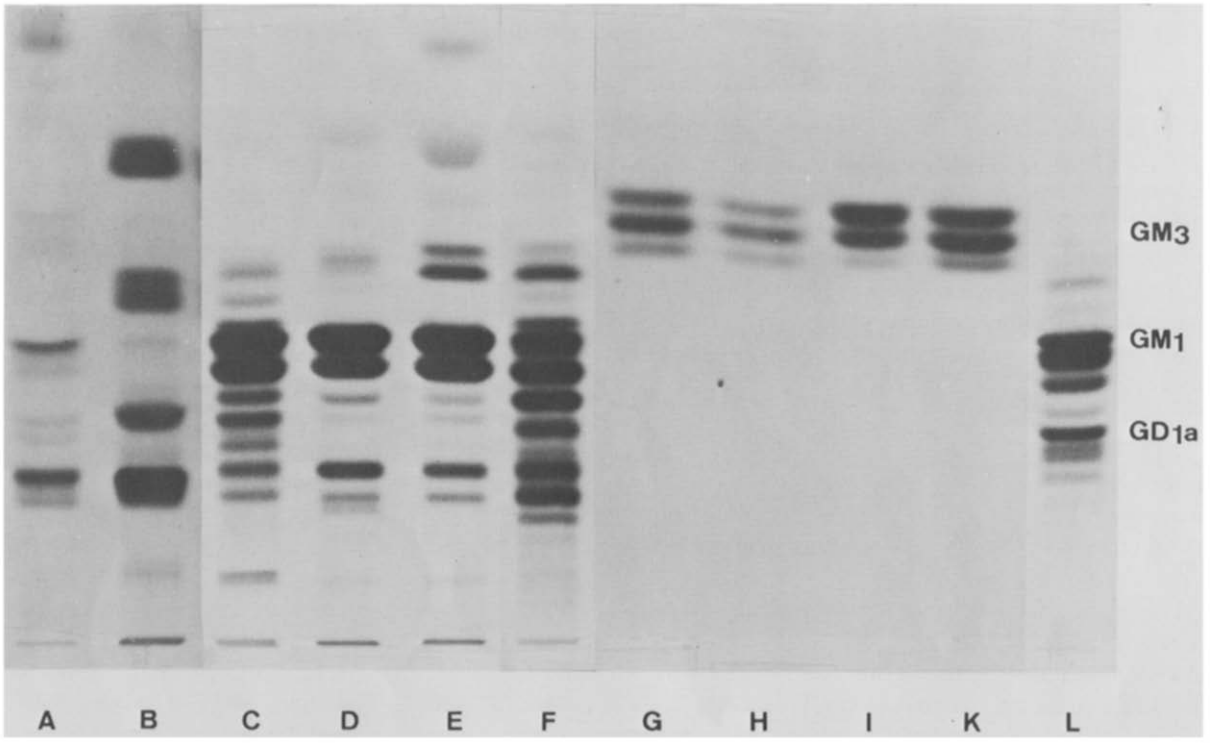
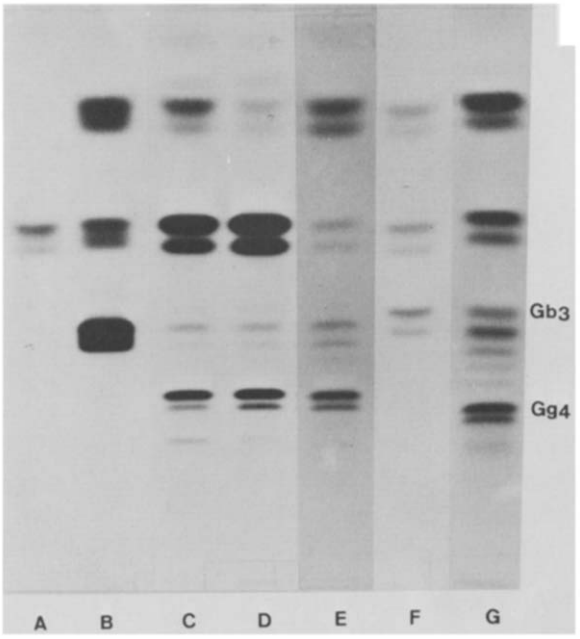


Fig.1. Silica gel HPTLC labelling patterns of gangliosides extracted from cell pellets of lymphoma cell lines (table 1) BW 5147 (A), EL-4 Bu (B), 344TC11 (C), T2D4 (D), E-7 (E), X63 (G), X63-Ag8.653 (H), Sp2/0 (I), and Sp1/HK (K), and from Con A (F) and LPS (L; from *Salmonella minnesota* R595)-stimulated DBA/2f Bom mouse spleen cells. About 5000 cpm of every sample were applied to HPTLC plates (Merck, Darmstadt) silica gel 60, size 10 × 10 cm, thickness 0.24 mm using an automatic sample applicator (Linomat III, CAMAG, Muttenz, Switz.). The plates were developed in a solvent system of chloroform:methanol:water (55:45:10, v/v/v, containing 1 mM $CaCl_2$). Autoradiography on Kodak RP Royal X-Omat X-ray films (exposure time 1 week) served for band identification. CBA/J mouse brain gangliosides chosen as cold reference substances were visualized by staining with resorcinol [14].



Gangliosides expressed by BW5147 and EL-4 are totally different, and thymoma 344TC11 is characterized by a third pattern, more similar to that of Con A-activated splenic T cells than those shown by the other two thymomas. The two T-cell hybridomas T2D4 and E-7, a clone of the T2D4 line [13], display largely overlapping patterns, partly resembling those of 344TC11 and Con A-activated cells. Clone E-7,

Fig.2. Silica gel HPTLC labelling patterns of neutral glycolipids extracted from cell pellets of lymphoma lines (table 1) BW5147 (A), EL-4 (B), T2D4 (C), E-7 (D), and from Sp1/HK (F), and from Con A (E), and LPS (G)-stimulated DBA/2f Bom mouse spleen cells. Application, autoradiography, and developing of the plates was as in fig.1, except that a solvent system consisting of chloroform:methanol:water (120:70:17, v/v/v) was used, and globotriaosyl ceramide of human erythrocytes and gangliotetraosylceramide of acid-treated human-brain gangliosides were applied as cold references, stained with orcinol [14].

Table 2
Quantitative evaluation of radioactivity present in ganglioside bands developed on Silica HPTLC plates (see fig.1)[a]

R_F value of bands[b]	Thymoma and T-cell hybridoma lines					Con A	Myeloma and B-cell hybridoma lines				LPS
	BW5147	344TC11	T2D4	E-7	EL-4		X63	X63-Ag8.653	Sp2/0	Sp1/HK	
2.03	5.3										
1.95	7.9			1.5							
1.81	3.0										
1.71	2.7			2.2		1.2					1.6
1.62					8.8						
1.56					17.0						
1.53		1.0					26.5	25.6	47.2	28.6	1.8
1.45							42.4	37.3	30.2	45.8	1.7
1.36							19.9	18.2	9.6	16.6	2.0
1.19		2.3		2.5	7.6	3.2					5.3
1.10		2.3			7.2						3.4
1.00		12.7				4.4					25.2
0.95	13.4	23.6	55.2	51.2	1.4	12.8					26.9
0.89	5.4	22.6	20.2	22.5		16.4					11.7
0.79		7.1	2.4	1.7		17.1					4.2
0.75	18.6	5.6	1.4	1.2	8.0	5.1					4.6
0.63	7.9	2.9		1.6		2.0					2.4
0.56		5.0	6.5	3.4	25.4	4.4					1.2
0.48		2.7	2.3	2.1		3.3					
0.41		1.2	1.4			1.6					
0.21		1.9									
yield	64.2	90.9	89.4	89.9	75.4	71.5	88.8	81.1	87.0	91.0	

[a] Areas of HPTLC silica gel corresponding to bands developed by ^{14}C-label on X-ray films were scraped off and counted in Aquasol (New England Nuclear, Dreieich). Percentages are based on total recoveries per sample in bands and background areas. This recovery was ~60–80% of applied radioactivity. Bands accounting for <1% were omitted

[b] R_F values were expressed relative to mouse brain ganglioside GM_1 = 1.00 as added standard

Table 3
Quantitative evaluation of radioactivity present in neutral glycolipid bands developed on silica HPTLC plates (see fig.2)[a]

R_F value of bands[b]	Thymoma and T-cell hybridoma lines					Con A	Myeloma and B-cell hybridoma lines			LPS
	BW5147	344TC11	T2D4	E-7	EL-4		X63	X63-Ag8.653	Sp1/HK	
2.00			1.6	1.1		5.0				
1.84			1.4	1.2						1.5
1.75	3.3	7.3	6.7	1.6	6.8	17.4	17.7	16.3	14.7	18.3
1.68	3.1	5.5	2.4	1.1	7.1	14.7	20.4	8.6	9.9	8.8
1.32	33.4	7.8	35.3	45.4	6.2	5.6	8.5	22.1	14.2	11.4
1.25	20.4	7.0	14.8	21.0	5.6	2.8	5.6	18.0	10.3	6.2
1.00			1.5	1.0			8.7	4.4	16.7	4.8
0.95	3.3	5.1	2.2	1.6	27.6	5.2	8.6	4.1	9.9	7.2
0.89	3.1	5.4	1.3	1.3	31.7	4.1				3.8
0.80										2.1
0.74										2.3
0.68	2.4	10.0	8.9	9.9	1.1	17.6	3.7	2.1		9.0
0.64			2.9	2.9		7.4	3.3	2.1		5.0
0.55		3.7	1.1			1.2				3.2
0.50										
yield	69.0	51.8	80.1	88.1	86.1	81.0	76.5	77.7	75.7	83.6

[a] Method as described in legend of table 2. Bands accounting for <1% were omitted

[b] R_F values were expressed relative to human erythrocyte triosylceramide I

appears to express somewhat greater variability than its parent line. The similarities between T2D4- and E-7-associated patterns extend to the neutral glycolipids, showing again the greatest overlaps with Con A-stimulated splenic T cells. Similarities are also seen with BW5147 neutral glycolipids (table 3). EL-4 thymoma cells of C57BL/6 mouse origin are very distinct from the above patterns.

4. Discussion

The differences in [^{14}C]galactose- and [^{14}C]glucosamine-labelled ganglioside and neutral-glycolipid distribution patterns observed upon HPTLC analysis of B and T lymphocytic tumour cell lines and hybridomas may reflect tumour-associated or functional properties, depending on the activities of the various enzyme systems involved in the metabolism of glycosphingolipids, e.g., sugar transferases and hydrolases, acyltransferases. The biochemical analysis of glycosphingolipids of lymphocytes appears to be a new and promising avenue of marker evaluation. It is for this reason that we undertook these studies.

Glycosphingolipids of cell surfaces are possible receptors for mitogen action [18,19]; they have been associated with Thy-1 alloantigens, and it has been suggested that they regulate the cell cycle and cell growth in immune responses [20]. Functional differences, e.g., of T cells, may, therefore, find their structural correlates in the nature of these constituents of eukaryotic cell membranes. In fact, we have suggestive evidence that glycosphingolipid patterns permit differentiation between T_H and cytotoxic T cells (G. R., M. H. Schreier, D. G. B., unpublished). The Fcγ-bearing T_S hybridoma T2D4 and E-7-associated glycosphingolipid patterns discussed here appear to resemble a third functional subset of T cells, rather similar to patterns of Con A-stimulated T cells. These were reported to constitute T suppressor (T_S) cells [2]. It is interesting that ganglioside patterns of T2D4 and E-7 cells bear little resemblance to that of BW5147 thymoma, although this line was used for fusion [13]. Both hybridomas, however, express strain AKR T cell-like patterns of the type shown by thymoma 344TC11 cells, i.e., ^{14}C-labelled bands are distributed over the area of GM1 (monosialo-) and GD1a (disialo-) gangliosides. T2D4 and E-7 are Fcγ-bearing T-cell hybridomas [13]. Therefore, 344TC11 cells may also represent a T_S cell population [22]. The evidence that Con A activates mainly T_S cells [20] would be compatible with the observed similarity of patterns, provided T cell-ganglioside distribution patterns can be associated with functional properties. On the basis of this reasoning, it is possible that thymoma line 344TC11 may also exert suppressive activity on immune responses. The distinct ganglioside and neutral glycolipid patterns of EL-4 cells may relate to functional differences associated with these T cells before they were transformed into tumour cells; they may also reflect genetic difference within the MHC.

Ganglioside patterns of the myeloma cell lines X63, X63-Ag8.635, Sp1/HK and Sp2/0 deserve special mentioning. Cell lines X63 and Sp1/HK still secrete immunoglobulins while cell lines X63-Ag8.653 and Sp2/0 do not [8–12]. Lines X63 and X63-Ag8.653 are myelomas, whereas lines Sp2/0 and Sp1/HK are hybridomas. Despite their different historical background, these 4 lines share the property that 80–90% of the [^{14}C]galactose and [^{14}]glucosamine cluster in the GM3 range. This area, however, contains only 5–6% of gangliosides expressed by polyclonal (LPS) B-cell stimulation (table 2). The inversion of band strength in line Sp2/0 is unlikely to be associated with the loss of immunoglobulin production by this line, since line X63-Ag8.653, also being a non-secretory B cell tumour, lacks it. However, this finding identifies a biochemical marker by which the two lines may be distinguished. It remains to be established whether ganglioside restriction to the GM3 class may be generally associated with plasma cells.

The isolation of specific glycosphingolipids on a preparative scale for further structural analysis and for the use in competition experiments is now feasible. In addition, highly specific anti-glycosphingolipid antibodies have recently been produced by the hybridoma approach [23]. These advances in glycosphingolipid research should soon be used to accumulate critical information about the functional properties of this category of membrane components. Tumour lines with certain pattern restrictions as well as specially identified hybridoma lines will be indispensible for the isolation of critical amounts of this material.

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