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Monoclonal antibody to galactosylceramide: discrimination of structural difference in the ceramide moiety

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A mouse monoclonal antibody (mAb) was developed against monohexaosylceramide This mAb differentially reacted on thin-layer chromatograms with 3 types of galactosylceramide (GalCer) obtained from bovine brain Structural analysis of the 3 glycolipids revealed that they consisted of the same galactose and sphingosine but of apparently different fatty acids Among the 3 GalCers, the mAb reacted with two GalCers which contained α-hydroxy fatty acids, but not with GalCer composed of nonhydroxy fatty acids These findings suggest not only that the mAb discriminated the fatty acid composition in the ceramide moiety of GalCer, but also that the ceramide structure defines the immunological epitope as it is known to do for the carbohydrate moiety of glycosphingolipid

Monoclonal antibody, Galactosylceramide, Ceramide, Immunological epitope, Thin-layer chromatography immunostaining

1. INTRODUCTION

Glycosphingolipids (GSL) which are present on the surfaces of animal cells have been well characterized structurally despite their molecular diversity [1–3]. These molecules are known to be involved in many membrane functions, such as those associated with cell growth, ontogenesis, differentiation and malignant transformation [1]. Furthermore, when added exogenously, they show potent biological activities in vitro or in vivo [4]. The advent of the monoclonal antibody (mAb) has produced much more knowledge of and attention to GSL [5,6]. Interestingly, most mAbs to GSL, when produced by immunization with cells or purified molecules as antigens, react with the nonreducing termini of the carbohydrate structures of GSL.

In the present report, we describe a mouse mAb which recognizes the immunological epitope composed of both carbohydrate and ceramide moieties of galactosylceramide.

2. MATERIALS AND METHODS

2 1. *Production of mAb*

The mAb was generated as described previously [7]. In brief, BALB/c mice were immunized with cultured human pancreatic cancer cells, designated as SUIT-2 The mAb-producing hybridomas were prepared by fusion of splenic cells from immunized mice and the mouse myeloma cell line, P3X63-AG-8-UI Hybridomas were further propagated in mice as intraperitoneal tumors The culture supernatant from the hybridomas or the ascites from tumor-bearing mice was used for immunological assays.

2.2. *Preparation of GSL*

GSL of SUIT-2 cells and standard glycolipids from bovine brain which was often used as a source of reference GSL were prepared as reported previously [8]. In brief, glycolipids were isolated by extraction with organic solvent and subsequent mild alkaline hydrolysis Glycolipids were then separated into neutral and acidic fractions by DEAE-Sephadex A-25 column chromatography Each GSL was further purified by Iatrobeads column chromatography.

2 3 *TLC and TLC-immunostaining*

Purified GSL were analyzed by TLC performed with a solvent system of chloroform/methanol/water (60:40:10, v/v) and visualized with orcinol/H_2SO_4 reagent which identifies the carbohydrates in GSL [8]. The immunoreactivity of the mAb with GSL was examined by TLC-immunostaining, as described previously [9]

2 4 *Elucidation of precise structure of CMH*

The compositions of the carbohydrate, fatty acid, and sphingosine components of CMH from bovine brain were determined by TLC, gas-liquid chromatography (GLC), and fast atom bombardment mass spectrometry (FAB-MS) as reported previously [8,10]

3. RESULTS AND DISCUSSION

A mAb (IgM subclass) was produced by one of 26 hybridomas which reacted with SUIT-2 cells and were partly described previously [7]. Since trypsin digestion did not alter the reactivity of the mAb with SUIT-2 cells (data not shown), we purified GSL from the cells to characterize the immunoreactive antigen. Fig.1 shows

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Abbreviations. TLC, thin-layer chromatography, mAb, monoclonal antibody, GSL, glycosphingolipid, CMH, monohexaosylceramide; GalCer, galactosylceramide

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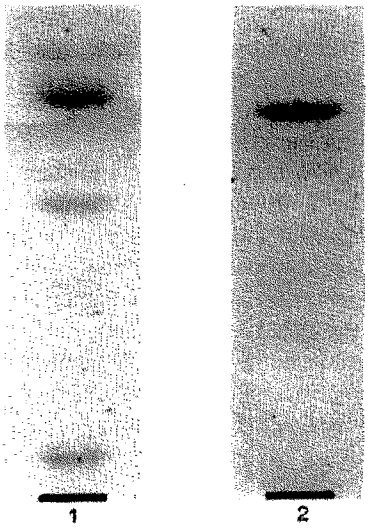

Fig.1. TLC (lane 1) and TLC-immunostaining (lane 2) of the total GSL purified from SUIT-2 cells.

both the TLC of GSL visualized with orcinol reagent (lane 1) and TLC-immunostaining with the mAb (lane 2). The mAb immunologically labeled only one of the orcinol-positive bands (lane 2 in fig.1). The labeled band appeared to be a CMH from its mobility on the TLC plate compared with the reference GSLs from bovine brain (data not shown). The amount of immunologically labeled GSL in the cells, however, was not great enough for structural analysis. We next examined the immunoreactivity of the mAb with standard CMH purified from bovine brain. Purified CMH showed 3 orcinol-positive bands (two major bands and one minor band) on a TLC plate (lane 1 in fig.2). Interestingly, the mAb reacted with the lower two of the 3 bands of CMH (clear and faint bands) by TLC-immunostaining (lane 2 in fig.2). There were no detectable bands following nonspecific background staining with preimmune serum in lieu of the mAb (lane 3 in fig.2). The individual CMH bands were then further separated. Two of them were clearly isolated but the lower one was not separated from the middle one, as shown in panel D in fig.2. Panel D shows orcinol staining of the isolated CMH, with an upper band (lane 4), a middle band (lane 5), and a mixture of middle and lower bands (lane 6). Panel E in fig.2 shows the immunoreactivity of the isolated bands with the mAb.

Analysis by GLC revealed that the GSL were all GalCers and that there was a striking difference in the fatty acid compositions (table 1). The immunoreactive GalCers (middle and lower bands) were composed of α-hydroxy fatty acids, whereas the immunonegative upper GalCer contained no α-hydroxy fatty acids. The lower GalCer seemed to predominantly contain α-hydroxy fatty acids with shorter chains as compared with those of the middle one. There was no difference in the other components (table 1). These findings were further confirmed by FAB-MS (data not shown).

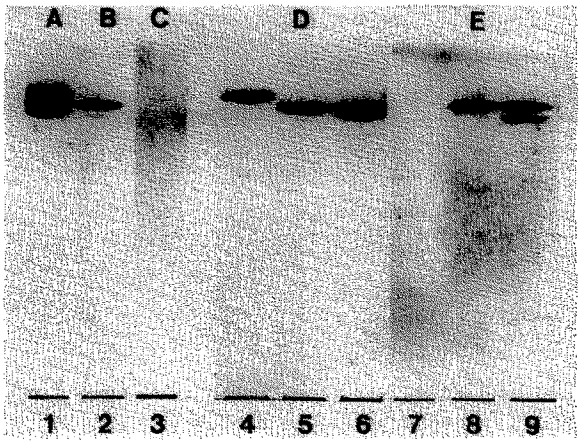

Fig.2. The immunoreactivity of CMH from bovine brain. TLC (panels A and D), TLC-immunostaining with mAb (panels B and E), and nonspecific staining with preimmune serum (panel C). (Lanes 1–3) total CMH; (lanes 4 and 7) isolated upper band of CMH; (lanes 5 and 8) middle band of CMH; (lanes 6 and 9) mixture of middle and lower CMH bands.

Thus, the mAb reacted with GalCers and discriminated the fatty acid composition in the molecules, while the usual mAb to GSL recognized the carbohydrate structures in the molecule [5,6]. Thus, the immunologically labeled GSL from SUIT-2 cells also might be a GalCer with α-hydroxy fatty acids.

The reactivity of the mAb with structurally related glycolipids was also examined as follows. Panel A in fig.3 shows TLC of galactosylsphingosine which was

Table 1

Structural analysis of CMH purified from bovine brain by GLC

	Upper	Middle	Middle + lower
Fatty acid			
18:0	2.68	–	–
20:0	0.56	–	–
22:0	5.78	–	–
23:0	7.30	–	–
24:1	34.92	–	–
24:0	26.68	–	–
25:1	8.58	–	–
25:0	7.89	–	–
26:0	5.51	–	–
18h:0	–	0.42	35.80
22h:0	–	0.62	0.33
23h:0	–	0.76	3.10
24h:1	–	69.50	50.87
24h:0	–	28.70	9.90
Long chain base			
16d:0	0.1	0.1	0.1
16d:1	0.5	0.4	0.5
18d:0	3.0	2.7	3.2
18d:1	96.4	96.8	96.2
Sugar			
Galactose	100.0	100.0	100.0
Glucose	0.0	0.0	0.0

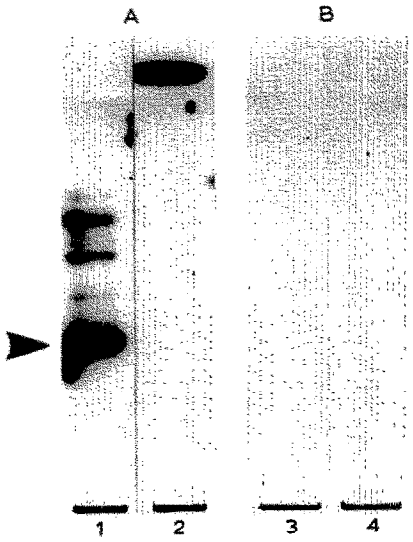

Fig.3. Immunoreactivity of two GalCer analogues. TLC (panel A) and TLC-immunostaining (panel B) of both galactosylsphingosine (lanes 1 and 3) and galactosyldiacylglycerol (lanes 2 and 4).

prepared from GalCer purified from human brain (indicated by an arrow in lane 1) and galactosyldiacylglycerol purified from porcine testis (lane 2). These GalCer analogues were not immunologically reactive on a TLC plate (lanes 3 and 4 of panel B in fig.3). These results suggested that the immunological epitope required both sphingosine and α-hydroxy fatty acids in the ceramide, i.e. the ceramide moiety also defined the immunological epitope, as it is well known to do in the case of the sugar moiety of GSL. Furthermore, the mAb labeled one of the GSL from SUIT-2 cells (fig.1) and also 2 of the 3 GalCer of bovine brain (fig.2), although they all contained the ceramide moieties. This result suggested that the epitope required the carbohydrate moiety in addition to the ceramide with α-hydroxy fatty acids.

We have reported a mouse mAb which reacted with 2 of 3 GalCer among GSL from bovine brain by TLC-immunostaining. This was of particular interest because of being the first mAb with well-characterized antigenic determinants in GalCer, and because of its discrimination of α-hydroxy fatty acids from nonhydroxy fatty acids in the molecule. The mAb was prepared using human cancer cells although GalCer has never been known to be a tumor antigen. This GSL is known to be a differentiation marker expressed on Schwann cells and oligodendrocytes in nervous system [11]. The development of this new mAb may provide further information about the functions of GalCer. Moreover, the role of hydroxylation of the fatty acids of the ceramide moiety in the antigenicity of GSL appears to be worth investigating because the ceramide moiety may define the immunological epitope even at the nonreducing carbohydrate terminal of GSL [12].

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