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A monoclonal antibody for terminal β-galactose. Use in analysis of glycosphingolipids

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A monoclonal antibody (mAb 8281) specific for the terminal β-galactose (βGal) of glycosphingolipids (GSL) and glycoproteins was produced from mice immunized with lipid extract from fresh acute lymphocytic leukemia (ALL) cells. Immuno-thin layer chromatography (ITLC) and competition assays with purified neutral GSL standards, free sugars, and synthetic neoglycoproteins showed mAb 8281 to be strongly reactive with LacCer, GalCer and Gal-β-*O*-$(CH_3)_2S(CH_3)_2$-CONH-(Gal-β-*O*-CETE) linked to bovine serum albumin (BSA). The penultimate sugar also played a role in binding. The antibody was not reactive with carbohydrates with terminal αGal structures and unrelated terminal moieties. Indirect immunoperoxidase staining and flow cytometry with mAb 8281 demonstrated positive staining on numerous tissues, including smooth muscle, gastrointestinal mucosa, lymph node B cells and monocytes. ITLC analysis of the GSL composition of fresh B cell neoplasms using mAb 8281 confirmed the presence of lactosylceramide and galactosylceramide in neoplasms of varying stages of differentiation. Because of its specificity for terminal βGal carbohydrate residues, mAb 8281 may be useful in structural and functional analyses of GSL.

Keywords: β-galactose, glycosphingolipids, monoclonal antibody

Terminal galactosyl structures on carbohydrates are important in a variety of biological systems. Galactose-specific receptors are found on murine hepatocytes, hepatic endothelial cells, and hepatic macrophages [1–3]. Galactose (Gal) receptors have also been demonstrated in murine bone marrow sinusoidal endothelium and in spleen; these galactose receptors are thought to be involved in the homing of marrow cells [4]. Other galactose-binding proteins have been implicated in immunorecognition in arthropods [5]. Anti-αGal antibody is present in human sera, regardless of blood type, in quantities up to 1% of total IgG [6] and has a postulated role in the removal of senescent [7] and aberrant [8] red blood cells from the circulation. Terminal αGal(1-3) glycoconjugates commonly found in other mammals are not common in primates [9]. These observations suggest a possible evolutionary response to primate gastrointestinal and pulmonary bacteria such as *Salmonella* and *Escherichia coli*, whose lipopolysaccharides have αGal(1-3) termini [9, 10]. In addition, some evidence exists to support a role for human natural anti-αGal antibodies in the immune surveillance of tumors; natural anti-αGal IgG has been shown to bind to some human malignant mammary tumors [11]. Anti-αGal antibody also mammary carcinoma cell lines and to cells of primary significantly inhibited tumor cells attachment in *in vitro* assays [11]. These data all point to the importance of exposed galactose termini as recognition signals for biological and immunological functions.

Many tumor-associated monoclonal antibodies (mAb) are directed against neutral glycosphingolipids (GSL), gangliosides, or glycoproteins whose antigen epitopes are oligosaccharides composed of various combinations of galactose, *N*-acetylgalactosamine, fucose, and neuraminic acid (SA) [12]. These carbohydrate structures have been implicated in cell–cell communication [13], in differentiation [14], and in the regulation of cell adherence properties which may contribute to the metastatic potential of tumors [15].

In the present report, we describe a mAb with binding specificity for terminal βGal-containing structures. Unlike previously described antibodies against terminal Galβ1-4Glcβ1-1Cer (LacCer) structures [16–18], mAb 8281 can bind specifically to the terminal βGal alone of neutral GSL, gangliosides, and synthetic neoglycoproteins. The chemical structures reactive with 8281 were analysed using galactose analogues and related carbohydrates. This unusual specificity for a single, common, terminal sugar moiety makes mAb 8281 a useful tool for carbohydrate structure identification. It may complement the current β-galactosidase experimental

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methods for identifying these structures because it does not modify the carbohydrate under study and therefore would enable analysis of the function of these important glycoconjugates in *in vitro* and *in vivo* systems.

Materials and methods

Antisera

Alkaline phosphatase-conjugated rabbit antisera specific for mouse IgM and IgG subclasses were purchased from Zymed (San Francisco, CA, USA) for use in enzyme-linked immunosorbant assays (ELISA). Rabbit antibodies to mouse IgG and IgM (Dako, Santa Barbara, CA, USA) were used in immuno-thin layer chromatography (ITLC). Rabbit antisera specific for IgM, IgG1, IgG2, IgG3 and IgA for use in Ouchterlony immunodiffusion assay were purchased in kit form from Serotec (Oxford, UK).

Glycosphingolipids and derivatives

Purified GSL used in high performance thin layer chromatography (HPTLC) and ITLC analyses were purchased from CalBiochem (La Jolla, CA, USA), Sigma (St. Louis, MO, USA), and Supelco (Bellefonte, PA, USA); these included glucosylceramide (GlcCer), galactosylceramide (GalCer), lactosylceramide (LacCer), globotriaosylceramide (Gb3), globoside (Gb4), GM1, GM2, GM3, GD1a, GD1b, and GT1b [Ganglioside names as per Svennerholm J (1964) *J Lipid Res* **5**:145–55.] Galβ1-3GalNAc-*O*-CETE-BSA was kindly provided by M. R. Suresh (Biomira, Edmonton, Alberta, Canada). GD3 and GD2 were obtained from melanoma cell line extracts and were kindly provided by Dr. H. Yuasa (Sloan-Kettering Institute, New York, NY, USA).

The GSL extract used for immunization was prepared by a modification of the methods of Hakomori [19] and Makita and Taniguchi [20]. Peripheral blood leukemia cells obtained by leukapheresis from a patient with common acute lymphocytic leukemia antigen positive acute lymphocytic leukemia (ALL) were extracted for 15 min in chloroform/methanol, 2/1 by vol, and twice in chloroform/methanol/H_2O, 10/10/1 by vol, each time with sonication for 15 min, prior to overnight extraction and 15 min sonication in chloroform/methanol/0.8% sodium acetate, 30/60/8 by vol. The total lipid extract was then saponified with KOH in methanol for 4 h at 40°C and dialysed against cold distilled water for three days to remove phospholipids. The extract was dried and stored at −70°C.

Hybridomas and monoclonal antibodies

BALB/c mice were immunized with the total GSL extract of leukemia cells from a patient with ALL (described above). The lipid extract was noncovalently absorbed to the *Salmonella minnesota* mutant R595 according to the method of Livingston *et al.* [21] by sonication in PBS. Half a milligram of R595 was mixed with extract derived from approximately 50–100 mg of packed leukemia cells. Each mouse was injected five times over four months with 100 µl of the lipid extract/R595 mixture. Spleen cells were harvested three days after the last immunization, and hybridomas were prepared [22] using the myeloma cell line SP2/0 [23] as a fusion partner.

Hybridomas were subcloned three times. Screening was done on the total lipid extract by ELISA. After sonication in phosphate-buffered saline (PBS), 0.2 µl/well of extract was dried onto Nunc 60 well Terisaki plates overnight at room temperature. Nonspecific binding was blocked by incubation with 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Supernatant was added for 1 h and then plates were blotted and washed three times with 0.1% BSA/PBS prior to incubation with a 1:200 dilution of rabbit anti-mouse IgG and IgM for 90 min. Plates were again blotted and washed three times with 0.1% BSA/PBS. Ten µl of a solution of one tablet of dinitrophenolamine (Sigma) suspended in 5 ml of 0.01% diethanolamine/0.005% $MgCl_2$, pH 9.8, were applied to each well and plates were incubated in the dark for 10 min at 37°C. Results were analysed on an Artek Systems 210 ELISA plate reader.

The isotype of the antibody was determined by ELISA and by Ouchterlony immunodiffusion assay (Serotec).

Antibody specificity

Reactivity of mAb 8281 against known and unknown purified neutral GSL and gangliosides was tested by ITLC according to a modification of the method of Magnani *et al.* [24]. Briefly, aluminum-backed silica HPTLC plates (precoated silica gel 60 F254, Merck) were activated at 100°C for 1 h. HPTLC was performed using known purified GSL, as well as GSL extracts from patient samples. The plates were then fixed in 0.05% polyisobutylmethacrylate (Aldrich, Milwaukee, WI, USA) in n-hexane at 40°C for 30 s and dried thoroughly. Nonspecific binding was blocked by incubation for 15 min in 1% BSA/PBS after spraying plates with the latter for 1 min. Plates were washed four times in cold PBS and incubated for 1 h at 0°C with the clarified hybridoma supernatant fluid. After re-washing with PBS, plates were reincubated for 1 h at 0°C with ^{125}I-labeled rabbit antimouse Ig (1 million cpm ml^{-1}, 5000 cpm ng^{-1} Ig), washed, and dried prior to exposure to Kodak (Rochester, NY, USA) film overnight at −70°C, using a Cronex (Sigma) intensifying screen.

Specificity was also tested in ELISA and in competition ELISA using purified free sugars (Pfanstiehl, Waukegan, IL, USA), synthetic neoglycoproteins (Carbohydrates International, Malmö, Sweden), and GSL. GSL were plated at 10 µg ml^{-1} and neoglycoproteins were plated at 5 µg ml^{-1}; ELISA were conducted as described above. Competition assays were done by incubating a dilution of hybridoma supernatant with varying concentrations of free sugars or neoglycoproteins overnight before proceeding with the

ELISA. Red cell agglutination assays were performed as previously described [25] at 0°C for 4 h.

The C-6 position of the terminal galactose of LacCer was oxidized to an aldehyde by galactose oxidase based on the method of Suzuki and Suzuki [26]. Twenty-five mg of LacCer in 50% 0.1 M sodium phosphate, pH 7.0, 50% tetrahydrofuran was reacted overnight at 24°C with 2 units of galactose oxidase (Sigma). After drying the reaction mixture under liquid nitrogen, the GSL products were obtained by phase separation in chloroform/ methanol/H_2O, 2/1/3 by vol.

Tissue distribution of the antigen

The distribution of binding of mAb 8281 on human tissue was determined by indirect immunoperoxidase staining on fresh-frozen tissues [27]. Distribution of mAb 8281 antigen on peripheral blood cells was determined by indirect flow cytometry [28] with fluorescein-conjugated goat anti-mouse antibody (Becton-Dickinson, Mountain View, CA, USA) using an Epics Profile flow cytometer.

Effector function

Complement mediated cytotoxicity assays were performed on cell lines in the following manner: 25 μl of cells at a concentration of 4–5 × 10^6 cells cc^{-1} were incubated with 25 μl of rabbit or human serum and 25 μl of antibody at 1:1 and 1:10 dilutions in round-bottom, 96 well plates for 50 min at 37°C. Cell death was determined using dye exclusion with Trypan Blue.

Radioiodination of antibodies

Radioiodination was done using $Na^{125}I$ (Amersham) by the chloramine-T method [29].

Results

Hybridoma production

Fusion of the SP2/0 cell line with spleen cells from immunized BALB/c mice initially yielded 654 clones; supernatant from 50 reacted with the immunizing material in the screening ELISA. After subcloning three times, four stable, reactive clones remained. Clone 8281 was selected for further study on the basis of the strength of its reactivity in ELISA against the starting material. In addition, mAb 8281 was strongly reactive in ELISA against GSL extracts from patients with chronic lymphocytic leukemia (CLL) and T cell ALL and showed less reactivity against red blood cell neutral GSL and gangliosides. ELISA and Ouchterlony immunodiffusion assay identified mAb 8281 as isotype IgM.

Antibody specificity

ITLC of mAb 8281 against known purified neutral GSL and gangliosides showed the strongest reactivity with LacCer and GalCer, both of which contain a terminal βGal structure (Fig. 1A); GalCer with different ceramide chains (I and II) were both reactive. Much weaker reactivity was also noted with ganglio-*N*-tetraosylceramide (asialo-GM1), as well as with gangliosides GM1 and GD1b (Fig. 1B), which share the same terminal βGal structure followed by an *N*-acetylated penultimate sugar (structures 4, 5, 6, Table 1). No reactivity was demonstrated with GSL having a terminal αGal, βGlc, or βGalNAc; specifically, no reactivity was seen with GlcCer, Gb3, or Gb4 (structures 8, 9, 10).

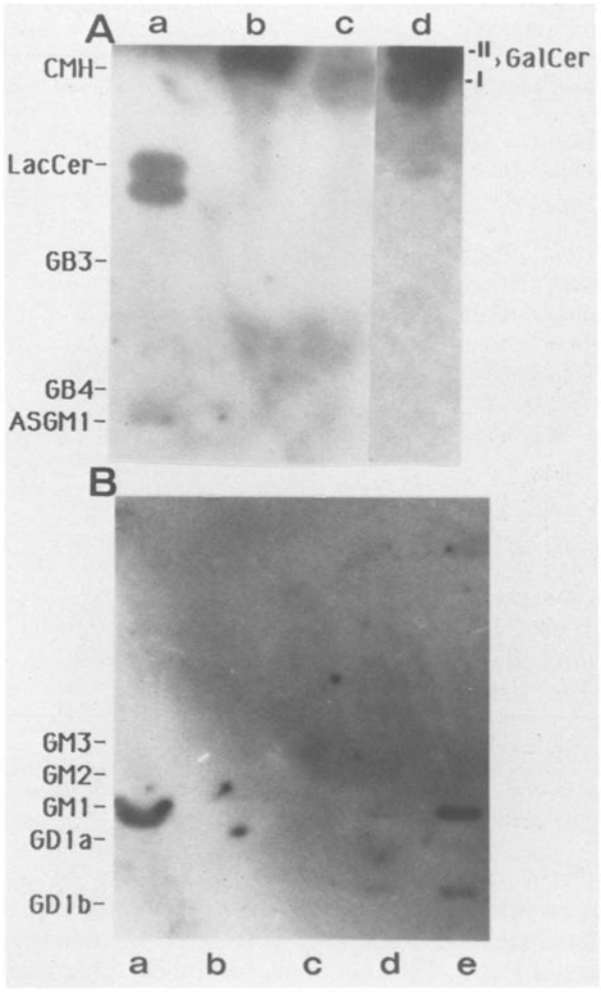

Figure 1. Immuno-thin layer chromatography of mAb 8281 with purified GSL standards. A: *Neutral GSL*; a) GlcCer, LacCer, Gb3, ASGM1, Gb4. b) Gal-II-Cer, Asialo-GM2. c) Gal-I-Cer. d) Gal-I- and Gal-II-Cer. B: *Gangliosides*; a) GM1. b) GM2. c) GM3. d) GD1a. e) Bovine brain ganglioside mixture containing GM1, GD1a, GD1b, GT1b. Only LacCer, GalCer, ASGM1, GM1, and GD1b stain positively with mAb 8281. (Plate B was exposed four times longer than Plate A). Migration of standards was shown on the left.

Table 1. Specificity analysis of mAb 8281. ITLC: mAb 8281 versus neutral GSL and gangliosides.

Carbohydrate structure[a]	*ITLC result*
1. Galβ1-4Glcβ1-1Cer	P[b]
2. Galβ1-1Cer I	P
3. Galβ1-1Cer II	P
4. Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer	W[c]
5. Galβ1-3GalNAcβ1-4(SA2-8SA2-3)Galβ1-4Glcβ1-1Cer	W
6. Galβ1-3GalNAcβ1-4(SA2-3)Galβ1-4Glcβ1-1Cer	W
7. Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer	W
8. Glcβ1-1Cer	–[d]
9. Galα1-4Galβ1-4Glcβ1-1Cer	–
10. GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	–
11. GalNAcβ1-4Galβ1-4Glcβ1-1Cer	–
12. GalNAcβ1-4(SA2-3)Galβ1-4Glcβ1-1Cer	–
13. (SA2-3)Galβ1-4Glcβ1-1Cer	–
14. (SA2-8SA2-3)Galβ1-4Glcβ1-1Cer	–
15. GalNAcβ1-4(SA2-8SA2-3)Galβ1-4Glcβ1-1Cer	–
16. (SA2-3)Galβ1-3GalNAcβ1-4(SA2-3)Galβ1-4Glcβ1-1Cer	–

[a] numbers refer to structures listed in text.
[b] P = strongly positive; band visible on ITLC after overnight exposure.
[c] W = weakly positive; band visible on ITLC only after 48–72 h exposure.
[d] – = no visible bands on ITLC.

Table 2. Specificity analysis of mAb 8281. ELISA of mAb 8281 versus GSL and neoglycoproteins.

Carbohydrate structure	*Binding*[a]
1. Galβ1-4Glcβ1-1Cer	P[b]
2. Galβ1-1Cer I	P
3. Galβ1-1Cer II	P
4. Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer	W[c]
9. Galα1-4Galβ1-4Glcβ1-Cer	–[d]
10. GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	–
17. Galβ1-*O*-CETE-BSA	P
18. Galβ1-4GlcNAc-*O*-CETE-BSA	W
19. Galα1-4Galβ1-4Glcβ1-*O*-CETE-BSA	–
20. Galα1-4Galβ1-4GlcNAcβ1-*O*-CETE-BSA	–
21. Glcβ1-1Cer	–
22. Media	–
23. GalNAcβ1-*O*-CETE-BSA	–
24. GlcNAcβ1-*O*-CETE-BSA	–
25. Galβ1-3GalNAcα1-*O*-Ser-BSA	–
26. CH_3-*O*-CETE-BSA	–

[a] Binding titer was defined as the last well with OD_{450} >2 times background.
[b] P (positive) = 1:8 → 1:64.
[c] W (weak) = reactive when undiluted only.
[d] – (negative) = no binding above background.

Table 3. Specificity analysis of mAb 8281. Competition ELISA.

Carbohydrate structure	*50% Competition* (μM)
17. Galβ-O-CETE-BSA	< 4
26. Glc	>50000
27. Gal	>50000
28. $GalNH_2$	>50000
29. GalNAc	50000
30. GalNAcβ1-4Galβ1-4Glc	> 1000
31. Galβ1-4Galβ1-4Glc	600
32. Galβ1-4Glc	180

Carbohydrate epitope analysis

Because initial ITLC data suggested specificity of mAb 8281 for terminal βGal structures, a series of ITLC studies (Table 1) and ELISA (Table 2) were performed using purified neutral GSL, gangliosides, free sugars, and synthetic neoglycoproteins. ELISA using GSL and BSA-linked sugars confirmed the βGal specificity; positive results were obtained using Galβ1-4GlcCer, GalCer I, GalCer II, and Galβ1-*O*-CETE-BSA (structures 1, 2, 3, 17). Weakly positive binding was seen with Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1Cer (asialo-GM1, ASGM1) and Galβ1-4GlcNAcβ1-*O*-CETE-BSA (structures 4, 18). Similarly, competition ELISA demonstrated binding inhibition only with the three carbohydrate structures sharing a terminal βGal: Galβ1-4Glc, Galβ1-4Galβ1-4Glc, and Galβ1-*O*-CETE-BSA (structures 32, 31, 17; Table 3). Carbohydrate structures sharing a terminal αGal moiety (structures 9, 19, 20) or unrelated terminal moieties (structures 8, 26) did not bind or compete for binding to mAb 8281. Free galactose did not compete for binding, presumably because the β linkage is critical. *N*-Acetylation of C-2 of galactose eliminated all binding (structures 10–12, 23). Modification of the third carbon with sialic acid (structures 13, 14) also prevented binding to mAb 8281. Glucose (structure 8), which differs from galactose only in the position of the hydroxyl group at C-4, was also not reactive. Following the oxidation at C-6 to an aldehyde by galactose oxidase, no binding of mAb 8281 could be demonstrated in ITLC with the oxidized product, while binding of the antibody to the unmodified portion of the reaction mixture remained intact (Fig. 2).

Although the intact βGal moiety was sufficient for binding, the penultimate sugar also greatly influenced binding affinity. A carbohydrate structure with terminal Galβ1-4Gal (structure 31) was five times less effective in competition assay than one with Galβ1-4Glc (structure 32), suggesting that the area of the βGal linkage on the second sugar was also important. Moreover, ganglio series structures containing β(1-3) linkages and *N*-acetylated second carbons on the second sugar were 10–30 times less reactive in ELISA and ITLC, indicating that this area partially influenced binding as well. In fact, mAb 8281 did not react at all in ELISA with the BSA-linked form of the T antigen (structure 25), which contains both a β1-3 terminal galactose linkage

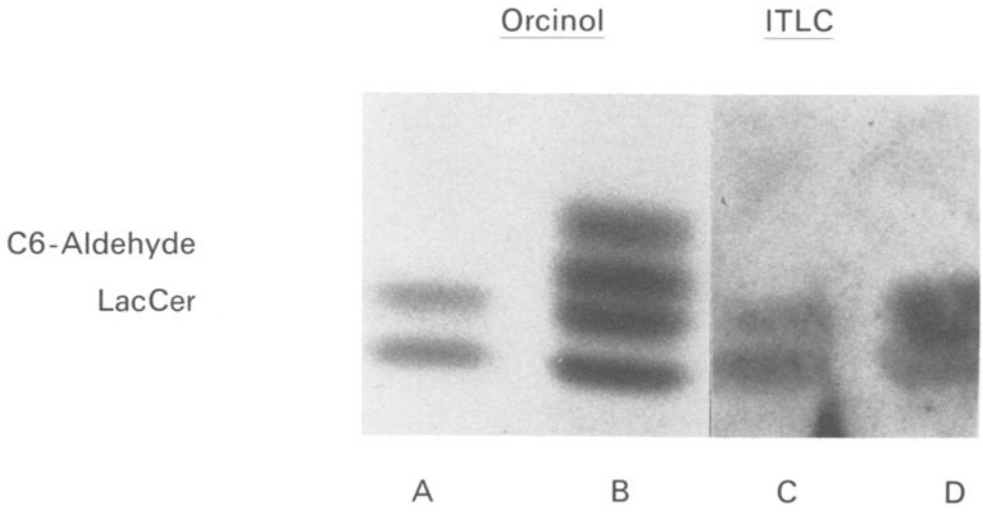

Figure 2. Galactose oxidase treatment of LacCer. Lanes A and B: Orcinol-stained HPTLC of LacCer (A) and reduction mix of LacCer and oxidized product containing aldehyde at C-6 (B). Lanes C and D: ITLC using mAb 8281 with LacCer (A) and reaction mix (B). MAb 8281 stains LacCer in both lanes. The oxidized product in Lane D does not bind the antibody.

and an *N*-acetylated galactose. This may be due to reduced affinity for this structure as well as to low density of carbohydrates on the BSA.

We cannot conclude from these data whether the sites described above actually participate in binding or whether structural changes at these sites prevent entry into the binding site or cause conformational changes elsewhere which affect binding.

mAb 8281 was used to identify GSL among B cell neoplasms using ITLC (Fig. 3). Both GalCer and LacCer were demonstrated in a number of samples; the expression of GalCer in non-neural cells is interesting. Paragloboside was also identified in acute lymphocytic leukemia cells. Because GalCer and GlcCer comigrate on HPTLC, mAb 8281 offers a simple method for distinguishing the expression of GalCer from the usual large expression of GlcCer.

Tissue distribution of the mAb 8281 antigen

Indirect immunoperoxidase staining of fresh-frozen tissues with mAb 8281 showed positive staining in numerous tissues, including smooth muscle, gastrointestinal mucosa, and lymph node B cells (Table 4). Indirect flow cytometry of two different samples of peripheral blood mononuclear cells showed reactivity with only a subset of monocytes (mean, 23%; range, 11–35%) (data not shown). Lymphocytes and polymorphonuclear cells were negative by flow cytometry. Since ITLC confirms the presence of the antigen in most peripheral blood cells, this implies that the antigen is cryptic in the majority of these cells. Red blood cell agglutination experiments were negative against $A+P_1+$, $A+P_1-$, $A-$, $B+$ and $O+$ red blood cells.

Table 4. Immunoperoxidase staining of fresh tissue with mAb 8281.

Organ	*Tissue*[a]	*Stain intensity*[b]
Kidney	Glomerulus	+/−
	Proximal tubule	+/−
	Collecting tubules	+
Stomach	Gastric mucosa	+
	Parietal cells	+/−
Esophagus	Epithelium	2+
Duodenum	Epithelium	2+
Ileum	Mucosa	+
Jejunum	Mucosa	+
Colon	Mucosa	+
Rectum	Mucosa	+
Lymph node	Germinal center	+
Liver	Parenchyma	+/−
Pancreas	Smooth muscle	+
Lung	Type 2 pneumocyte	+
Bladder	Mucosa	+
Cerebellum	Granular cell layer	+
	White matter	2+
Prostate	Epithelium	+
Breast	Alveolar ducts	+
Uterus	Myometrium	+
	Endometrium	+
Testis	Nerve fibers	+
Vagina	Epithelium	2+

[a] Smooth muscle was positive in all tissues tested; endothelium was negative.

[b] −, No staining; +/−, occasional positive cells; +, >50% positive cells; 2+, diffuse, strong staining.

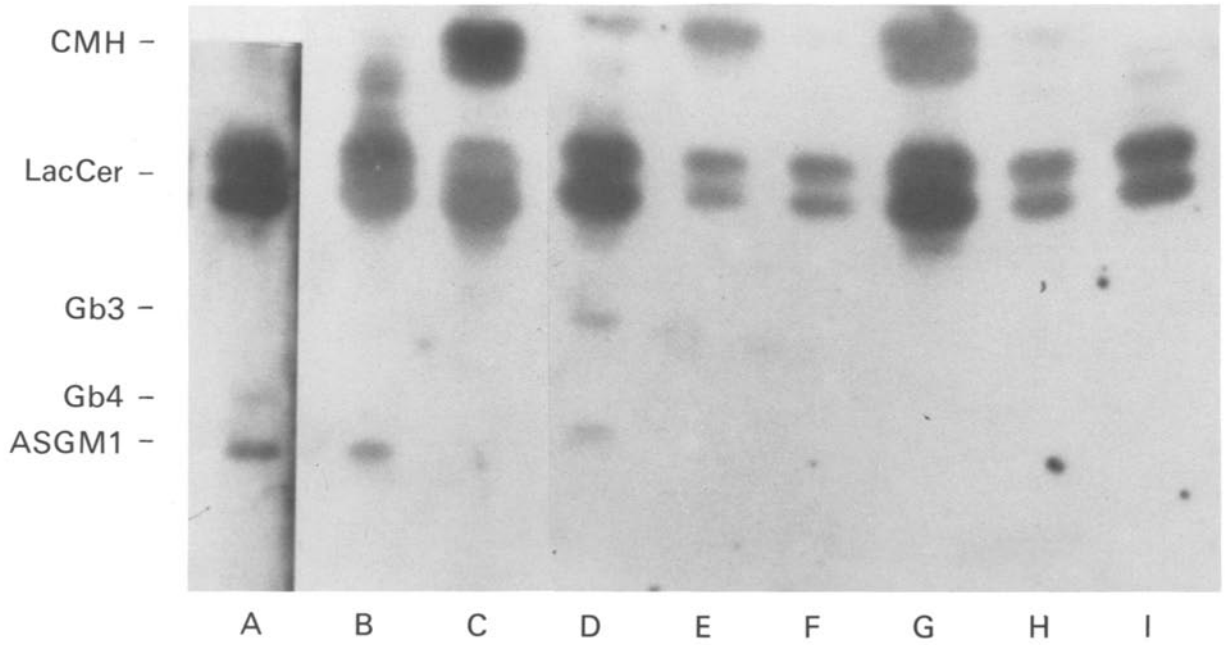

Figure 3. Identification of GalCer, LacCer and paragloboside in a variety of B cell neoplasms. ITLC analysis of neutral extracts and standard as follows: A: Acute lymphocytic leukemia (ALL). B: ALL. C: Standards including GalCer, LacCer, Gb3, Gb4. D: Multiple myeloma. E: Chronic lymphocytic leukemia (CLL). F: CLL. G: Standards as in C. H: Diffuse lymphoma. I: ALL. LacCer is seen in all samples; GalCer is seen in lanes B, D, E and markers; paragloboside is seen in lanes A and B. The migrations of standards are shown on the left. Paragloboside was not included as a standard, but migrates with ASGM1.

ITLC were also performed against neutral GSL extracts obtained from a variety of cell lines and from fresh tissue samples of B cell neoplasms. The distribution of the mAb 8281 antigen target in these samples is summarized in Table 5.

Effector function

In complement-mediated cytotoxicity assays using rabbit and human serum, no killing above background could be demonstrated against the cell lines SKLY 16, MOLT-4, Raji or Daudi. Similarly, killing was not demonstrated using peripheral blood mononuclear cells. However, significant killing above background was seen using the Burkitts lymphoma cell line Ramos as a target. This cell line had shown GalCer and LacCer reactivity with mAb 8281 in ITLC. Using human serum as a complement source, killing was demonstrated at a mean of 38% over background. Strong reactivity of 8281 was confirmed with live Ramos cells, but not with several other lymphoid cell lines in rosetting assays (data not shown). Ramos also reacted with mAbs to LacCer, suggesting that LacCer may be one possible target for the cytotoxicity. Radioimmunoprecipitation experiments of ^{35}S-labeled Ramos cell extracts showed no precipitation of protein, suggesting that the target may be a GSL, either LacCer or GalCer. Because the antigen was present in biochemical analyses of many cell lines but complement cytotoxicity was negative except in Ramos, this suggests (as do the flow cytometry data above) that the antigen is generally cryptic in live cells.

Table 5. 8281 ITCL data: B cell neoplasms and cell lines.

Sample	*LacCer*	*GalCer*	*Asialo-GM1*
Diffuse histiocytic lymphoma 1172	+		
Chronic lymphocytic leukemia (CLL) 272	+	+	
CLL 1113	+		
CLL 1248	+		
Acute lymphocytic leukemia (ALL) 1202	+		
ALL 004	+		
ALL 1183[a]	+		
Diffuse poorly differentiated lymphoma 1025	+		
Ramos[b]	+	+	
SKMM-2[c]	+		+

[a] Initial immunizing material.
[b] Burkitts lymphoma cell line.
[c] Multiple myeloma cell line.

Discussion

MAb 8281, an IgM antibody specific for terminal βGal-containing structures, was produced by immunizing mice with total GSL extract from cells of a patient with B cell ALL. Since prior studies have shown that the predominant neutral GSL in B cell ALL is LacCer [30], which contains a terminal βGal, it is not surprising that this specificity was selected. Of note is the fact that previously obtained antibodies to LacCer have been produced after immunization with acute nonlymphocytic leukemias [16–18]. Since our initial report [30], a second mAb specific for GalCer has been described [31]. Interestingly, this other antibody could discriminate GalCer with differences in ceramide composition; a detailed analysis of its carbohydrate reactivity has not been presented.

ITLC data, ELISA, and competition ELISA with a large number of oligosaccharides, GSL, and neoglycoproteins, as well as modification of the galactosyl antigen epitope with galactose oxidase treatment, showed consistent specificity of mAb 8281 for intact terminal galactose including the β linkage. These studies also showed the penultimate sugar to be influential in the degree of binding; antibody binding to Galβ1-4Glc, for example, was far stronger than to Galβ1-4GlcNAc, Galβ1-4GalNAc, or Galβ1-3GalNAc. Galα(1-4) linkages were not recognized by mAb 8281. Therefore, the type of penultimate sugar or its linkage may modify the efficacy of binding through conformational changes, steric hindrance, or other means.

The specificity of mAb 8281 for terminal βGal-containing carbohydrates makes it a potentially important tool in the study of exposed galactose termini. The simple, rapid identification of terminal galactose demonstrated by mAb 8281 in ITLC and ELISA suggests that this antibody will also be useful in identifying the structure of carbohydrates. We have already used mAb 8281 to identify the basic carbohydrate structures GalCer and LacCer in studies of the neutral GSL of B cell neoplasms at varying stages of differentiation [30]. GalCer was found in a number of samples, an unusual finding outside of the central nervous system [20]. Currently, the most common method for the determination of βGal termini is the use of β-galactosidase [31], with subsequent analysis of the products of the reaction. Since the use of mAb 8281 for terminal βGal detection does not result in enzymatic modification of the carbohydrate under study, broader applications of the antibody as a structural probe may be feasible. The ITLC data show that use of mAb 8281 reliably differentiates between GalCer and GlcCer, single-sugar neutral GSL which often co-migrate on HPTLC and are therefore difficult to distinguish. ITLC of mAb 8281 and GSL extracts from a variety of B cell neoplasms consistently differentiated GalCer from GlcCer.

Flow cytometry data showed reactivity of mAb 8281 with a minority of peripheral blood cells, specifically, a fraction of monocytes. This was unexpected in view of the fact that GSL extraction and subsequent ITLC confirmed the presence of the antigen in a majority of peripheral blood cells, and LacCer has been shown to be a major component of lymphocytes, monocytes, and polymorphonuclear cells (PMN) [16]. Similarly, complement-mediated cytotoxicity could not be demonstrated with peripheral blood mononuclear cells, even though a percentage of these cells stained positive by flow cytometry. These data indicate that the targets of mAb 8281 are cryptic and that the antibody may also be of interest in the study of antigen crypticity.

Crypticity may be the result of masking of the antigen at the cell surface by larger or more charged GSL or glycoproteins [33]. Symington *et al.* have shown evidence for an alternative mechanism for crypticity [34]; by use of the LacCer specific antibody T5A7, they have demonstrated the presence of intracellular LacCer antigen in the granules of PMN, instead of masking of antigen at the cell membrane. The reason for the crypticity of the mAb 8281 antigen is currently unclear and remains to be studied. Antibodies directed at LacCer have been designated the CDW17 leukocyte cell surface antigen cluster [18]. The antibodies in this group react with monocytes, PMN, platelets, basophils, and some acute nonlymphocytic leukemia cells [16, 18, 34]. MAb 8281 may also be part of this cluster group. However, although strongly reactive with LacCer, the flow cytometry studies of PMNs with mAb 8281 showed little binding, in contrast to the studies with T5A7. This may be due to differences in affinity between the two antibodies. The reactivity of mAb 8281 with acute leukemias, both lymphocytic and nonlymphocytic, will be studied in an attempt to clarify the differences between its antigen epitope and those of other CDW17 antibodies.

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