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Establishment of a monoclonal antibody directed against Gb_3Cer/CD77: a useful immunochemical reagent for a differentiation marker in Burkitt's lymphoma and germinal centre B cells

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A new monoclonal antibody (TU-1) directed against the Galα1-4Galβ1-4Glc residue of the Gb_3Cer/CD77 antigen was prepared by the hybridoma technique following immunization of mice with an emulsion composed of monophosphoryl lipid A, trehalose dimycolate, and Gb_3Cer isolated from porcine erythrocytes. TU-1 showed reactivity towards Gb_3Cer and lyso-Gb_3Cer (Galα1-4Galβ1-4Glcβ1-1′Sph), although the reactivity towards lyso-Gb_3Cer was about 10-fold lower than that to Gb_3Cer. But it did not react with other structurally-related glycolipids, such as LacCer (Galβ1-4Glcβ1-1′Cer), Gg_3Cer, Gg_4Cer, Gb_4Cer (GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1′Cer), galactosylparagloboside (Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1′Cer), sulfatide (HSO_3-3Galβ1-1′Cer), other gangliosides (GM_3, GM_2, GM_{1a}, GD_{1a} and GT_{1b}), or P_1 antigen (Galα1-4Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1′Cer) among neutral glycolipids prepared from P_1 phenotype red blood cells. Furthermore, TU-1 reacted with viable lymphoma cells, such as human Burkitt lymphoma cell line, Daudi, and Epstein-Barr virus (EBV)-transformed B cells by the immunofluorescence method, and also with germinal centre B cells in human tonsil and vessel endothelial cells in human thymus histochemically. These results indicate that TU-1 is a monoclonal antibody directed against Gb_3Cer/CD77 antigen and can be utilized as a diagnostic reagent for Burkitt's lymphoma and also for detection of the blood group P^k antigen in glycolipid extracts of erythrocytes.

***Keywords*: mouse monoclonal antibody; Gb_3Cer/CD77 antigen; P_1 antigen; Burkitt's lymphoma; germinal centre B cells**

***Abbreviations*: ATL, adult T-cell leukaemia; BSA, bovine serum albumin; Cer, ceramide; DPPC, L-α-dipalmitoylphosphatidylcholine; EBV, Epstein-Barr virus; FCS, fetal calf serum; GalCer, Galβ1-1′Cer; GlcCer, Glcβ1-1′Cer; LacCer, Galβ1-4Glcβ1-1′Cer; Gb_3Cer, Galα1-4Galβ1-4Glcβ1-1′Cer; lyso-Gb_3Cer, Galα1-4Galβ1-4Glc1-1′Sph; Gb_4Cer, GalNAcβ1-3Galα1-4Galβ1-4Glc1-1′Cer; galactosylparagloboside, Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1′Cer; Gg_3Cer, GalNAcβ1-4Galβ1-4Glcβ1-1′Cer; Gg_4Cer, Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1′Cer; GM_3, Neu5Acα2-3Galβ1-4Glcβ1-1′Cer; GM_2, GalNAcβ1-4(Neu5Acα2-3) Galβ1-4Glcβ1-1′Cer; GM_{1a}, Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glcβ1-1′Cer; GD_{1a}, Neu5Acα2-3Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glcβ1-1′Cer; GD_{1b}, Galβ1-3GalNAcβ1-4(Neu5Acα2-8Neu5Acα2-3)Galβ1-4Glcβ1-1′Cer; GT_{1b}, Neu5Acα2-3Galβ1-3GalNAcβ1-4(Neu5Acα2-8Neu5Acα2-3) Galβ1-4Glcβ1-1′Cer; HRP, horseradish peroxidase; LDH, lactate dehydrogenase; MAb, monoclonal antibody; MPL, monophosphoryl lipid A; P_1 antigen, Galα1-4Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1′Cer; PVP, polyvinylpyrolidone; Sph, sphingosine; sulfatide, HSO_3-Galβ1-1′Cer; TDM, trehalose dimycolate; TLC, thin-layer chromatography**

Introduction

The glycosphingolipid known as globotriaosylceramide (Galα1-4Galβ1-4Glcβ1-1′Cer or Gb_3Cer) has been implicated as an important functional ligand in several biological processes. Expression of Gb_3Cer is known to be elevated in various lymphomas [1, 2], leiomyosarcomas [3], seminoma [4], and familial dysautonomia [5]. Gb_3Cer has been identified as a ligand for Shiga [6, 7] and Shiga-like toxins [8, 9], and also as the P^k blood-group antigen [10]. This antigen is expressed on many types of human blood cells, such as erythrocytes [11], platelets [12], and

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lymphocytes [13]. In addition, the P^k structure accumulates in certain malignant lymphomas, *ie* Burkitt's lymphoma of the Epstein-Barr virus (EBV)-positive African type and the European/North American EBV-negative Burkitt-like lymphoma [1]. This structure is present on the cell surface and can be used as a marker for this particular B-lineage tumour [13, 14]. This antigen accumulation seems to be related to a particular differentiation stage in B-lymphocytic development, since treatment with differentiation-inducing agents such as butyrate and phorbol esters causes a decrease in the expression of this marker along with changes in other differentiation-related markers [15]. Recently Gb_3Cer was identified as a differentiation antigen for B lymphocytes (*ie* CD77), which was proposed as a marker for apoptosis of germinal centre B-cells [16, 17] and was shown to induce apoptosis in Burkitt lymphoma cells [18].

Several monoclonal antibodies to Gb_3Cer [13, 14, 19, 20] were produced by immunization with whole cells, natural glycoconjugates, or synthetic oligosaccharides by coupling them to an effective protein carrier for use as immunogens. Two such monoclonal antibodies, the epitopes of which were examined in detail, were frequently used as auxiliary reagents to CD77 antigen for diagnosis and classification of B-cell lymphomas and leukemias by flow cytometric analysis [13, 14, 16–20]. Rat monoclonal antibody (MAb) 38.13 [14], which was produced by immunization of rats with a Daudi Burkitt lymphoma cell line, is the most frequently used. This antibody recognizes the dissacharide structure Galα1-4Galβ1- as its epitope. Mouse MAbs 424/3D9 and 424/6A2 [20] were produced by immunization with synthetic neoglycoproteins containing (Galα1-4Galβ1-4Glcβ1-O and Galα1-4Galβ1-4GlcNAcβ1-O residues, and these antibodies showed specificities for trisaccharide structures Galα1-4Galβ1-4Glc(NAc) as epitopes. However, all of these above MAbs showed cross-reactivity to P_1 antigen (Galα1-4Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1′Cer) among neutral glycolipids prepared from P_1 phenotype red blood cells.

Recent reports have demonstrated that Gb_3Cer/CD77 antigen on Burkitt lymphoma cells may be associated with a transmembrane protein that can transduce an apoptoic signal perceived by the glycolipid [18, 21, 22]. Therefore, the establishment of monoclonal antibodies directed against Gb_3Cer/CD77 antigen, which can recognize epitopes distinct from those previously reported, would provide useful reagents to investigate the biological and physiological functions *in vivo* and *in vitro* of this glycolipid antigen.

Materials and methods

Preparation of glycolipids

All natural glycolipids used were purified in our laboratory from the following sources: Sulfatide, GM_{1a}, GD_{1a}, GD_{1b}, GT_{1b}, and GalCer, from bovine brain [23, 24]; GlcCer, LacCer, Gb_3Cer, and Gb_4Cer, from porcine erythrocytes [25]; GM_3 from human liver [26]; GM_2, from Tay-Sachs brain [27]; Gg_4Cer and Gg_3Cer, from guinea pig erythrocytes [28, 29]; and IV^3Neu5Ac-nLc_4Cer and galactosylparagloboside, from human red blood cells [30]. nLc_4Cer was prepared from IV^3Neu5Ac-nLc_4Cer by sialidase treatment as described previously [31]. Lyso-Gb_3Cer was prepared from Gb_3Cer as described previously [32]. The extraction and preparation of neutral glycolipids from blood group P_1 erythrocytes followed standard procedures. After chloroform/methanol extraction and Folch's partitioning, the water-soluble material (enriched in glycolipids with long carbohydrate chains) was prepared as described previously [33]. Concentrations of gangliosides and glycolipids were quantified by densitometric analysis using oricinol-HCl or resorcinol staining as described earlier [34].

Preparation of liposomes

Cholesterol (Sigma, Chemical, St Louis), L-α-dipalmitoylphosphatidylcholine (DPPC; Nippon Fine Chemicals, Osaka) were obtained commercially. Liposomes containing Gb_3Cer for immunization were prepared by a method described previously [35]. Briefly, L-α-dipalmitolyphosphatidylcholine (0.5 μmol), cholesterol (0.5 μmol), *Salmonella minnesota* R595 (10 μg) lipopolysaccharides, and Gb_3Cer (0.05 μmol), each dissolved in chloroform/methanol (2 : 1 by vol), were mixed in a conical flask. After evaporation of the solvent by means of a rotary evaporator and then a vacuum pump, 0.5 ml of PBS (pH 7.2) was added; and the mixture was dispersed by vigorous vortexing. The cholesterol/DPPC liposomes containing Gb_3Cer were used as immunogen for the production of MAbs as described below.

Production of monoclonal antibody

BALB/c mice (8 weeks) were immunized intraperitoneally with Gb_3Cer absorbed to monophosphonyl lipid A (MPL) and trehalose dimycolate (TDM) emulsion (RIBI adjuvant) by the procedure described in the instruction of the kits provided by RIBI (Hamilton, MT). Every mouse received intraperitoneal injections of the Gb_3Cer-MPL-TDM complex according to the following schedule: 10 μg of Gb_3Cer on day 0, 20 μg on day 5, and 20 μg on day 10. After a rest period of 2 weeks, each mouse received a final booster injection with 30 μg of Gb_3Cer. As another immunogen, cholesterol/DPPC liposomes in PBS containing Gb_3Cer and *S. minnesota* R595 lipopolysaccharides was used. Immunization with the lyposomes containing Gb_3Cer was performed by the method described previously [35]. Three days after the final injection, the spleen cells were harvested and fused by means of 30% polyethylene glycol 4000 (Sigma) with mouse myeloma X63-Ag8-6.5.3 cells that had been maintained in RPMI 1640 medium containing 10% fetal calf serum (GIBCO) [36]. Hybrid cells were selected in RPMI 1640 medium containing hypoxanthine,

aminopterin, and thymidine [37]. After 2–3 weeks of incubation, the culture supernatant of the hybrid cells was screened by the enzyme-linked immunosorbent assay (ELISA) described below. The hybridoma clones whose culture supernatants were positive only for MAb against Gb_3Cer were selected and closed twice by the limiting dilution method.

Assessment of reactivity of monoclonal antibodies with various glycolipids by enzyme-linked immunosorbent assay

Titres of the monoclonal antibody were assayed by ELISA as follows: antigen solution containing 0.1–100 pmol (usually 50 pmol) of Gb_3Cer was added to each well of 96-well Limbro (flat bottom) plates. The solvent was evaporated at 37 °C for 1 h, after which 100 µl of blocking solution [5% bovine serum albumin in PBS (BSA-PBS)] was added; and the plates were then kept for 2 h at room temperature. Next, the culture supernatant (50 µl) of each hybridoma was added to a well, and the plates were incubated for 1 h at room temperature. After five washes with PBS, 50 µl of second antibody, *ie* goat anti-mouse IgG/M conjugated with horseradish peroxidase (HRP; Pel-Freez, Arkansas), at a 1 : 1000 dilution, was added to each well; and the plates were kept for 2 h at room temperature. The wells were then washed five times with PBS and the plates were kept at room temperature after addition of substrate solution consisting of 2 mg of *ortho*-phenylenediamine (Nacalai Tesque, Kyoto) and 2 µl of hydrogen peroxide in 5 ml of citrate phosphate buffer (pH 7.0). After incubation of the plates for 15 min at room temperature in the dark, the reaction was stopped with 3 N hydrochloric acid, and the absorbance at 492 nm was measured using the absorbance at 630 nm as a reference.

TLC immunostaining of various glycolipids with monoclonal antibody

TLC immunostaining was performed with various glycolipids according to the methods described previously [35]. Briefly, glycolipids were developed on a thin-layer plate (Polygram, Sil G, Macherey-Nagel, Germany) with a solvent system of chloroform : methanol : water (65 : 35 : 8, by vol). After chromatography of various neutral glycolipids and gangliosides, the plate was dried and then kept in solution A (PBS containing 1% egg albumin, 1% polyvinylpyrolidone [PVP], and 0.02% sodium azide) at 4 °C overnight. After five washes with PBS, the plate was loaded with the culture supernatants of hybridomas and incubated for 2 h at 37 °C. The chromatogram was washed five times with PBS, and then incubated with PBS containing horseradish peroxidase-conjugated goat anti-mouse IgG/M antibodies (1 : 1000) for 2 h at room temperature. The plate was then washed five times with PBS and incubated with peroxidase substrate solution. The quantity of the reaction product was measured by reading the optical density at 578 nm with a dual-wavelength TLC scanner (CS-9000, Shimadzu, Kyoto).

Immunofluorescence staining and flow cytometric analysis

Immunofluorescence staining was performed with the use of a Cytoron Absolute flow cytometer (Orto-Clinical Diagnostics K.K., Tokyo) as described [38]. Cells were first incubated in a washing buffer [PBS, pH 7.4, 1% fetal calf serum (FCS), and 0.1% sodium azide] containing 5% normal goat serum and then treated with mouse monoclonal anti-Gb_3Cer antibody (IgM) or isotype-matched control mouse antibody at a concentration of 5 µg ml^{-1} for 30 min on ice. These cells were twice washed with the washing buffer, and subsequently stained with a 1 : 400 diluted FITC-labelled goat anti-mouse IgM antibody (Southern Biotechnology Associates, Inc, Birmingham, AL) for 30 min on ice. The stained cells were twice washed with the washing buffer, and then subjected to flow cytometric analysis.

Immunohistological staining

Tonsil and thymus were obtained from children undergoing tonsillectomy and cardiac surgery, respectively. Each tissue was embedded in OCT compound (LabTek Division, Miles Laboratories, Inc., Naoerville, IL) and then sectioned by a cytostat. The frozen 5 µm sections were mounted on gelatin- and egg albumin-coated glass slides, briefly fixed in chilled acetone, and stored at −20 °C. Immediately before staining, the frozen sections were re-fixed in cold acetone at 4 °C for 10 min, air dried, and immersed in PBS. The sections were overlaid with 20 µl of 5 µg ml^{-1} of mouse monoclonal anti-Gb_3Cer antibody (TU-1) for 60 min. Subsequently, immunoenzymic staining was performed by the alkaline phosphatase : anti-alkaline phosphatase (APAAP) procedure as described previously [39]. APAAP complexes were purchased from DAKO JAPAN (Kyoto, Japan), and the immunostaining was done according to the instructions provided by the company. These sections were counterstained with Carrazi's Hematoxylin and mounted in glycerol gelatin (Sigma Chemical, St Louis).

Lacate dehydrogenase (LDH) release from Daudi cells by treatment with TU-1

A solution containing 0.1 µg of rat anti-mouse IgM monoclonal antibody (CALTAG Laboratories, Inc., San Francisco, CA) in 50 µl of 0.25% BSA-PBS was added to each well of 96-well (flat bottom) Maxisorp Nunc-Immuno plates, and then the plates were incubated at 4 °C for 24 h. After the incubation, each well was washed three times with PBS, and further incubated in 100 µl of blocking solution (5% BSA-PBS) for 24 h at 4 °C. Daudi cells (1×10^5 cells per 100 µl) were incubated with 1 µg ml^{-1} and 0.1 µg ml^{-1} of TU-1 or isotype-matched control mouse antibody in RPMI

1640 medium containing 0.25% FCS (0.25% FCS-RPMI) for 30 min at 37 °C, and then the cells were washed twice with 0.25% FCS-RPMI. The pretreated cells (5×10^4 cells per 100 μl of 0.25% FCS-RPMI) were added to the wells immobilized with rat anti-mouse IgM monoclonal antibody or without the MAb, and further incubated for 16 h at 37 °C. After the incubation, LDH activity in each culture medium was examined as described previously [40]. Briefly, the medium (12.5 μl) was diluted four-fold with 100 mM Tris-HCl (pH 8.2) and mixed with 50 μl of the reagent containing 2 mM β-nicotinamide adenine dinucleotide (oxidative form), 200 mU ml^{-1} of dipharose, 190 mM lithium lactate, and 0.78 mM nitroblue tetrazolium in 100 mM Tris-HCl (pH 8.2). The mixture was incubated at 37 °C for 10 min, and the reaction was stopped by the addition of 0.5 N HCl (100 μl). The absorbance was measured at 550 nm (reference 630 nm).

Cell lines and cultures

Red blood cells of the P_1 phenotype were obtained from The Japanese Red Blood Cross Central Blood Center (Tokyo, Japan). Mouse myeloma X63-Ag8-6.5.3 cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 25 mM HEPES, and 100 U ml^{-1} penicillin. Burkitt lymphoma-derived cell line Daudi, myeloma cell line IM-9, T cell line MOLT-4, myeloid cell line HL-60, erythroblastoid cell line K562, and monocytoid cell line U937 were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). EBV-transformed B cell lines, *ie* TM, ST, and CY, were established from normal donors as previously described [41]. Adult T-cell leukemia (ATL) lines HUT-102 and MT-2, were generously supplied by Dr T. Uchiyama (Institute for Virus Research, Kyoto University, Kyoto, Japan). Another T-cell line, Jurkat, was provided by Dr K. Yamamoto (Cancer Research Institute, Kanazawa University, Kanazawa, Japan). These cell lines were maintained in RPMI 1640 medium supplemented with 10% FCS, 25 mM HEPES, 100 U ml^{-1} penicillin, 10 μg ml^{-1} gentamicin, and 3 mg ml^{-1} amphotericin B.

Results and discussion

Production of the hybridoma

Fusion of X63-Ag8-6.5.3 mouse myeloma cells with spleen cells of mice immunized against Gb_3Cer-MPL-TDM complex (RIBI adjuvant) yielded 8.0% positive clones of hybrid cells in the first assay after fusion. These positive wells were examined for their reactivity towards LacCer, GlcCer, and simple lipids such as cholesterol and DPPC. Twelve hybridomas had specific reactivity towards Gb_3Cer in the second assay. Furthermore, these hybridomas were characterized for their reactivity towards Gb_4Cer, and five of the 12 produced antibodies specifically against Gb_3Cer. Of those five hybridomas U-1 and Q-II to Gb_3Cer showed reactivities much higher than those of the others, and these hybridomas showed good growth. In addition, the culture supernatant of these hybridomas did not react with P_1 antigen (Galα1-4Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1′Cer) among the neutral glycolipids extracted from P_1 phenotype red blood cells by the thin-layer chromatography/enzyme immunostaining procedure (data not shown). Hybridoma U-1, the most potent secretor of anti-Gb_3Cer antibody, was selected and cloned by the limiting dilution method. On the other hand, fusion of mouse myeloma cells with spleen cells from mice immunized with liposomes containing Gb_3Cer yielded 7.7% positive clones of hybrid cells in the first assay. However, all of these positive clones showed reactivity towards simple lipids such as cholesterol and/or DPPC, and no clones reactive only to Gb_3Cer were obtained. Presumably, as the antigenicity of neutral glycolipids as immunogen would be lower than that of gangliosides containing *N*-acetylneuraminic acid, the use of an adjuvant for immunization might be necessary to obtain a monoclonal antibody with high specificity towards a neutral oligosaccharide residue. Our results indicate that immunization using the RIBI adjuvant system, as employed in this study, might be more effective than that using the liposome procedure reported previously [35] to obtain monoclonal antibodies directed against oligosaccharide residues on natural neutral glycolipids.

Specificity of the monoclonal antibody directed against Gb_3Cer

The reactivity of the monoclonal IgM(κ) antibody, TU-1, produced by the hybridoma U-I, to various neutral glycolipids and gangliosides was determined by ELISA (Figure 1a). It is clear that the TU-1 MAb was reactive only towards Gb_3Cer. Structurally-related neutral sphingolipids, such as LacCer (Galβ1-4Glcβ1-1′Cer), Gb_4Cer (GalNAcβ1-3Galα1-4GAlβ1-4Glcβ1-1′Cer), galactosylparagloboside (Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1′Cer), and other neutral glycolipids, such as Gg_4Cer and Gg_3Cer, showed no reaction with TU-1. Furthermore, ganglio-series gangliosides containing *N*-acetylneuraminic acid, *ie* GM_3, GM_2, GM_{1a}, GD_{1a}, GD_{1b}, GT_{1b}, sialylparagloboside (IV^3Neu5Ac-nLc_4Cer), and other acidic glycosphingolipids, such as sulfatide, were also negative towards the antibody. In addition, Figure 1b shows that Gb_3Cer reacted with culture supernatant of TU-1 depending on the amount of the antigen (0.5–100 pmol), as determined by ELISA. The lower limit of detection of Gb_3Cer by TU-1 in the ELISA was about 0.1 pmol per well. Next, the specificity of TU-1 monoclonal antibody was further tested by the thin-layer chromatrography/enzyme immunostaining procedure. A positive reaction was obtained only with Gb_3Cer, and no reactivity was observed with natural neutral glycolipids (GalCer, GlcCer, LacCer, Gb_4Cer, paragloboside, Gg_3Cer, Gg_4Cer) or with GM_{1a}, GM_2, GM_3, GD_{1a}, and GT_{1b}

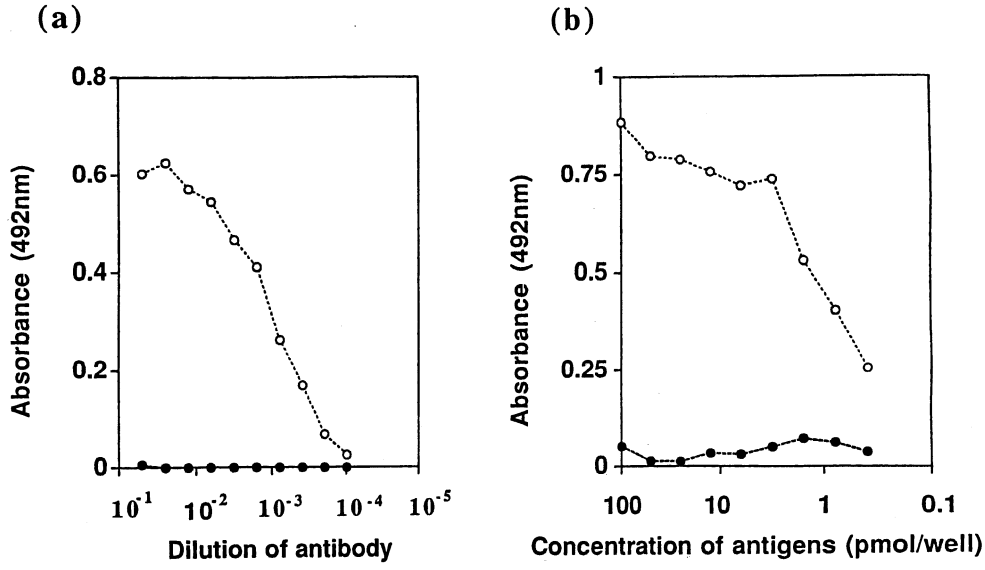

Figure 1. Reactivity of monoclonal antibody TU-1 with various neutral and acidic glycolipids determined by ELISA. (a) The reactivity of TU-1 was examined with various neutral and acidic glycolipids at 50 pmol per well. The activity was determined with various concentrations of hybridoma culture supernatant. (b) Dose dependency for various glycolipids of hybridoma culture supernatant was examined. Various concentrations (0.5–100 pmol per well) of glycolipids were added to each well and the reactivity of the culture supernatant towards each was analysed. ○–○, Gb_3Cer; ●–●, GalCer, GlcCer, LacCer, Gb_4Cer, galactosylparagloboside, Gg_3Cer, Gg_4Cer, GM_3, GM_2, GM_{1a}, GD_{1a}, GD_{1b}, GT_{1b}, sulfatide.

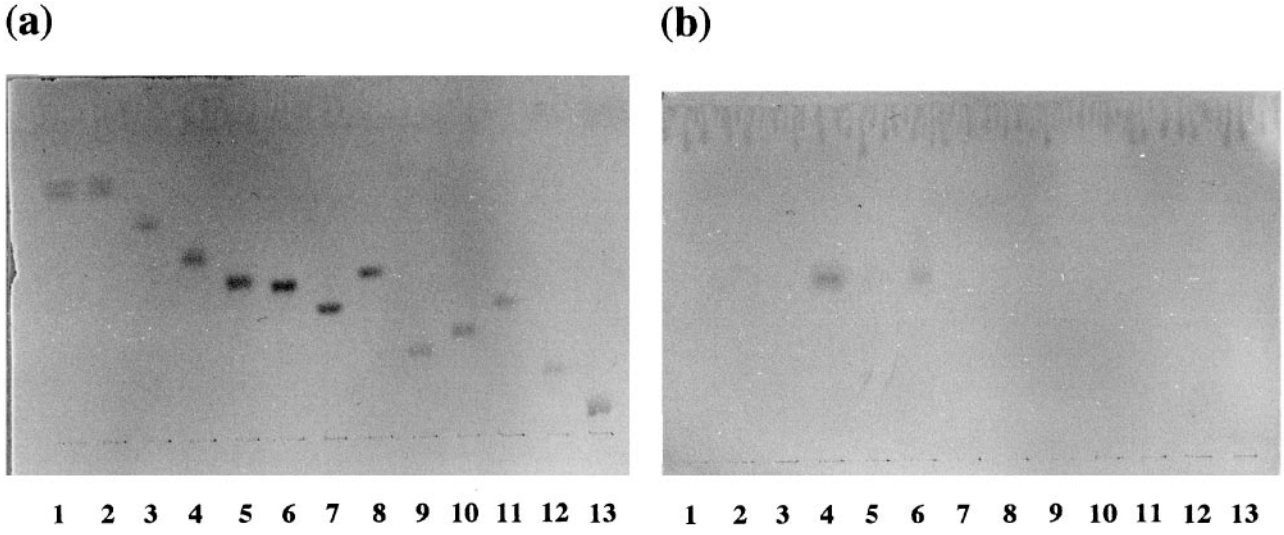

Figure 2 Reactivity of monoclonal antibody TU-1 with various neutral glycolipids and related glycolipids as determined by the TLC/Immunostaining procedure. Various glycolipids (500 pmol per lane) were applied to silica gel thin-layer plates, developed in chloroform : methanol : 12 mM $MgCl_2$ (55 : 45 : 10, by vol), and analysed by the thin-layer chromatograpy/enzyme immunostaining method as described in the text. Each antigen on the plate was reacted with the supernatant containing TU-1. After incubation for 2 h at 30 °C, the plate was stained by horseradish peroxidase-conjugated goat anti-mouse IgG/M antibody solution (1 : 1000) and 4-chloro-1-naphtol substrate solution. (a) oricinol-HCl staining; (b) TLC/Immunostaining. 1, GalCer; 2, GlcCer; 3, LacCer; 4, Gb_3Cer; 5, Gb_4Cer; 6, nLc_4Cer; 7, Gg_4Cer; 8, Gg_3Cer; 9, GM_{1a}; 10, GM_2; 11, GM_3; 12, GD_{1a}; 13, GT_{1b}.

(Figure 2b). No reactivity was observed either with galactosylparagloboside, sialylparagloboside, or sulfatide by this procedure (data not shown). In addition, the sensitivity of TU-1 for detection of Gb_3Cer was examined by the thin-layer chromatography/enzyme immunostaining procedure. TU-1 reacted with Gb_3Cer depending on the amount of the antigen (0.5–100 pmole) by the procedure, and the lower limit of detection of Gb_3Cer by the antibody was about 1 pmole per lane. The lack of reactivity towards LacCer, Gb_4Cer, galactosylparagloboside, which contain Galβ1-4Glc-,

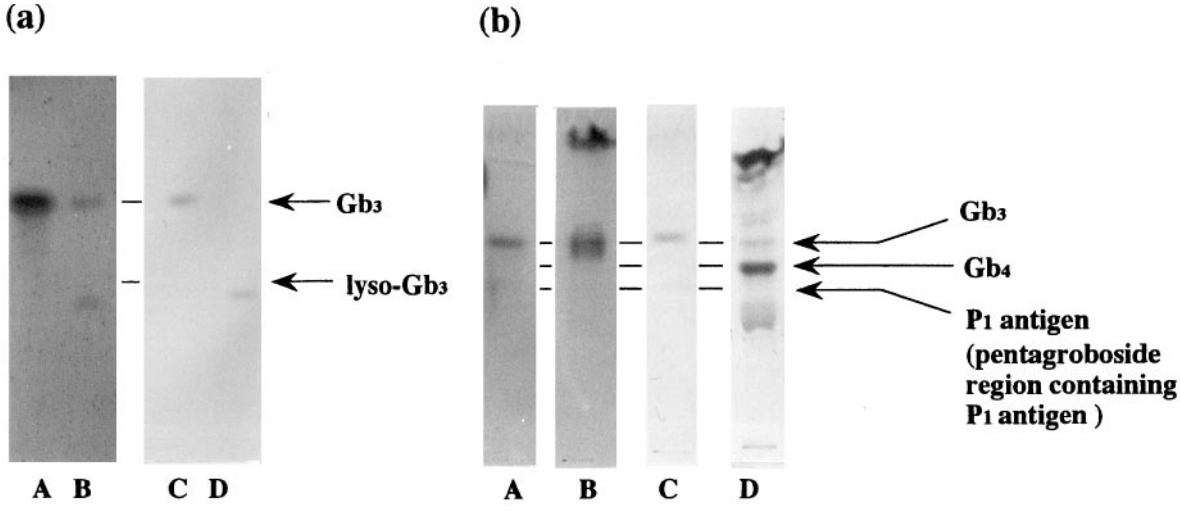

Figure 3. Immunochemical staining procedure using TU-1 MAb for staining of lyso-Gb_3Cer and neutral glycolipids from P_1-erythrocytes separated by thin-layer chromatography. (a) Immunochemical staining using the TU-1 MAb from the culture supernatant and purified Gb_3Cer (300 pmol per lane) (lane A) or lyso-Gb_3Cer (300 pmol per lane) (lane B) separated by the thin-layer chromatography. The same glycolipids were stained with orcinol-HCl on a chromatogram from a parallel run (lanes C and D). The 300 pmol of lyso-Gb_3Cer solution contained about less than 10 pmol of Gb_3Cer, and this amount of Gb_3Cer was below the level of detection by orcinol-HCl staining. (b) Immunochemical staining using the TU-1 MAb from the culture supernatant and purified Gb_3Cer (300 pmol per lane) (lane A) or neutral glycolipids (30 μg per lane) from P_1-erythrocytes (lane B) separated by thin-layer chromatography. The same glycolipids were stained with orisinol-HCl on a chromatogram from a parallel run (lanes C and D). The reaction of the front position in lane B was non-specific staining by the second antibody, as the simple lipids were stained with the second antibody alone.

GalNAcβ1-3Galα1-4Galβ1-4Glc-, and Galα1-3Galβ1-4GlcNAc- residues, respectively, indicate that the antibody does not recognize structurally-related integral oligosaccharide (Galα1-4Gal) or Galα1-3Galβ1-4Glc(NAc) terminal sugar residue.

Reactivity of TU-1 towards lyso-Gb_3Cer, neutral glycolipids of P_1 phenotype red blood cells

To further examine the epitope of TU-1 in detail, we examined the reactivities of TU-1 towards lyso-Gb_3Cer and P_1 antigen (Galα1-4Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1′Cer) among the neutral glycolipid fraction of P_1 phenotype human red blood cells by the thin-layer chromatography/enzyme immunostaining procedure. As shown in Figure 3a, TU-1 could react with lyso-Gb_3Cer, although the reactivity in this case was about 10-fold lower than that towards Gb_3Cer. Lyso-Gb_3Cer reacted with TU-1 depending on the amount of the antigen (5–100 pmol). On the other hand, as shown in Figure 3b, TU-1 reacted with Gb_3Cer (P^k antigen), but not with the pentaglycolipid region containing P_1 antigen (Galα1-4Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1′Cer) among neutral glycolipids from P_1 phenotype human red blood cells. The above results indicate that TU-1 recognizes Galα1-4Galβ1-4Glc residue and a part of the ceramide structure as the epitope. The antigen specificity of this antibody tested in this study is summarized in Table 1. Previously described MAbs, such as 38.13 [13, 14] and 424/3D9 [20], were reported to react with P_1 antigen among neutral glycolipids from P_1 phenotype red blood cells. Furthermore, these MAbs recognize terminal Galα1-4Gal sugar residues [14] and Galα1-4Galβ1-4Glc(NAc) residues [20] as their epitopes, respectively. These results clearly indicate that the epitope defined by TU-1 is different from that of 38.13 or 424/3D9 and that TU-1 is a unique monoclonal antibody exclusively reactive with Gb_3Cer.

Immunofluorescence staining of viable cells analysed by flow cytometry

To examine the reactivity of TU-1 MAb towards viable cells, we carried out indirect immunofluorescence on various cell lines including Burkitt's lymphoma by flow cytometry. As summarized in Table 2, TU-1 bound to the Burkitt lymphoma Daudi cell line. By immunofluorescence more than 50% of Daudi cells were clearly stained with undiluted hybridoma supernatant containing TU-1. The antibody gave a clear but weaker staining with three EBV-transformed cell lines, TM, ST, and CY, and with a monoblastic lymphoma cell line, U937. No staining, however, was obtained for T lymphoma cell lines (Molt-4 and Jurkat), a lymphoblastoid B cell line (IM-9). ATL cell lines (HUT-102 and MT-2), an erythroblastoid cell line (K562), or a myeloid cell line (HL-60). These results are consistent with results reported previously [1, 13, 14, 16], indicating that TU-1 can also be utilized as an immunochemical reagent for detection of Gb_3Cer/CD77 antigen on viable cells obtained from patients with Burkitt's lymphoma or leukaemia.

Table 1. Carbohydrate structure of glycolipids and summary of the reactivity of TU-1 as ascertained by the ELISA and TLC/Immunostaining.

Glycolipid	*Structure*	*Reactivity*	
		ELISA	*TLC*
GalCer	Galβ-1′Cer	–	–
GlcCer	Glcβ-1′Cer	–	–
LacCer	Galβ1-4-Glcβ-1′Cer	–	–
Gb_3Cer	Galα1-4Galβ1-4Glcβ-1′Cer	+	+
lyso-Gb_3Cer	Galα1-4Galβ1-4Glcβ-1′Sph	+	+
Gb_4Cer(Globoside)	GalNAcβ1-3Galα1-4Galβ1-4Glcβ-1′Cer	–	–
nLc_4Cer (Paragloboside)	Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-1′Cer	–	–
Galα1-3nLc_4Cer (Galactosylparagloboside)	Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-1′Cer	–	–
Galα1-4nLc_4Cer (P_1 antigen)	Galα1-4Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-1′Cer	–	–
Gg_4Cer(Asialo-GM_1)	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ-1′Cer	–	–
Gg3Cer(Asialo-GM_2)	GalNAcβ1-4Galβ1-4Glcβ-1′Cer	–	–
GM_{1a}	Galβ1-3GalNAcβ1-4(Neu5Acα2-3) Galβ1-4Glcβ-1′Cer	–	–
GM_2	GalNAcβ1-4(Neu5Acα2-3) Galβ1-4Glcβ-1′Cer	–	–
GM_3	Neu5Acα2-3Galβ1-4Glcβ-1′Cer	–	–
GD_{1a}	Neu5Acα2-3Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glcβ-1′Cer	–	–
GD_{1b}	Galβ1-3GalNAcβ1-4(Neu5Acα2-8Neu5Acα2-3)Galβ1-4Glcβ-1′Cer	–	–
GT_{1b}	Neu5Acα2-3Galβ1-3GalNAcβ1-4(Neu5Acα2-8Neu5Acα2-3)Galβ1-4Glcβ-1′Cer	–	–
Sulfatide	HSO_3-3Galβ-1′Cer	–	–

Table 2. Expression of Gb_3Cer/CD77 antigen on human cell lines by flow cytometric analysis with anti-Gb_3Cer MAb.

Cell line	*Cell lineage*	*CD77 expression*
Daudi	Burkitt lymphoma cell	+ + + [a]
TM	EBV-transformed B cells	+
ST	EBV-transformed B cells	+
CY	EBV-transformed B cells	±
IM-9	B cells	–
MOLT-4	T Cells	–
JURKAT	T Cells	–
HUT-102	ATL line	–
MT-2	ATL line	–
K562	Erythroblastoid	–
HL-60	Myeloid	–
U937	Monocytoid	±

[a] Results are expressed as percentages of surface fluorescent cells relative to the total viable cell population: $<1\%$,–; 1–5%, ±; 5–20%, +; 20–50%, + +; $>50\%$, + + +.

Detection of Gb_3Cer in human tonsil and thymus by immunohistochemical examination

The experiments using frozen tonsillar tissues showed that a small fraction (10–20%) of tonsillar B cells were brightly stained with TU-1 but that T cells were not reactive with this antibody (Figure 4a). In addition, peripheral blood lymphocytes were not stainable with the antibody in cytocentrifuged preparations (data not shown). Immunochemical evaluation revealed that the TU-1 stained most of the cells in the germinal centre of the tonsil, whereas it reacted with only a few cells in the interfollicular area, and seemed to react with some vessels. On the other hand, in human thymus, vessel endothelial cells and also some epithelial cells were strongly stained with this antibody. It is well known that Gb_3Cer/CD77 antigen is expressed on the tonsillar germinal centre B lymphocytes [16]. The tissue distribution of the antigen has also been studied [19], however, the existence of Gb_3Cer and its distribution in human thymus have not been examined, although He *et al.* [42] reported Gb_3Cer to be present in rabbit, sheep and pig thymus. Our current results indicate that Gb_3Cer exists in human thymus and also that the antigen is distributed on vessel endothelial cells and on some epithelial cells in the thymus. Recent reports suggest that the Gb_3Cer/CD77 antigen could be involved in the triggering of apoptosis in tonsillar germinal centres [17,18] and that the antigens expressed on Burkitt lymphoma cell lines and Vero cells function as a receptor for Shiga and Shiga-like toxins [21,22]. The biological functions of the antigen in the thymus remain to be clarified. The distribution of Gb_3Cer/CD77 antigen on the vessel endothelial cells and on some vessel epithelial cells in the thymus may suggest that the antigen there could possibly function as a receptor for Shiga and Shiga-like toxins.

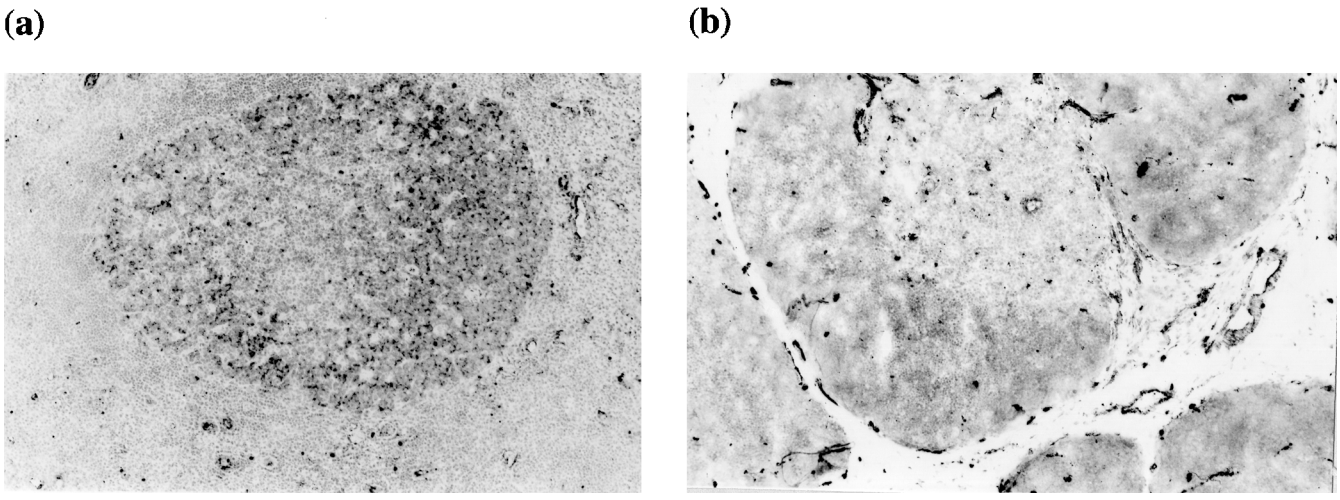

Figure 4. Distribution of Gb_3Cer/CD77 antigen in human tonsil and thymus. Distribution of the antigen was detected immunohistochemically by the alkaline phosphatase : anti-alkaline phosphatase (APAAP) procedure. Original magnification: × 100. (a) distribution of Gb_3Cer/CD77 antigen in human tonsil; (b) distribution of Gb_3Cer/CD77 antigen in human thymus.

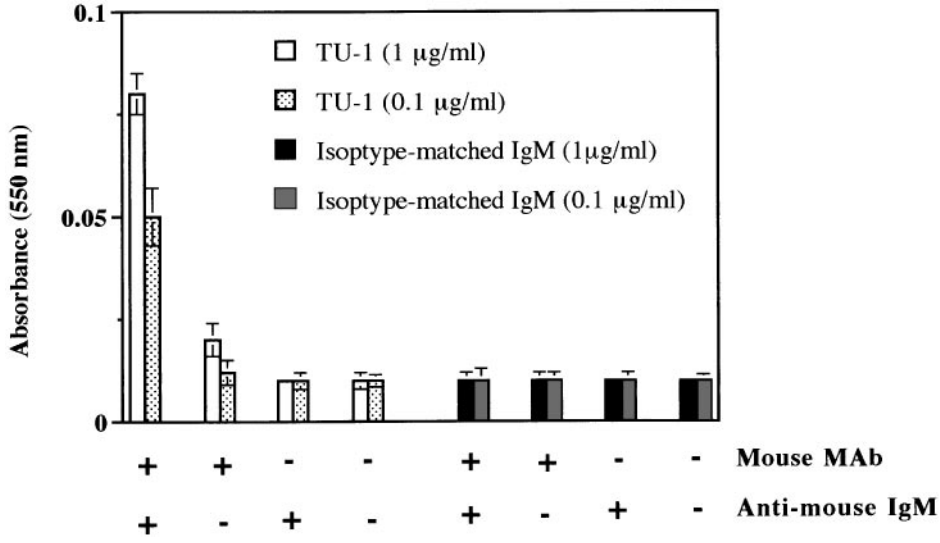

Figure 5. Cytolysis of Daudi cells by TU-1 MAb. Daudi cells (1×10^5 cells) were incubated with TU-1 (1 μg ml^{-1} or 0.1 μg ml^{-1}) or isotype-matched IgM (1 μg ml^{-1} or 0.1 μg ml^{-1}) for 30 min at 37 °C, and then the cells were washed twice with 0.25% FCS-RPMI. The pretreated cells (5×10^4 cells per 100 μl of 0.25% FCS-RPMI) were added to wells previously coated or not with anti-mouse IgM MAb (0.1 μg ml^{-1}), and further incubated for 16 h at 37 °C. After the incubation, LDH activity in each culture medium was measured as described in Materials and methods. The experiments were done in triplicate, and the results are presented as the mean ± SD.

Cytolytic activity of TU-1 towards Daudi cells

Mangeney *et al.* [18] demonstrated that a recombinant B-subunit of verotoxin (VT-B), which carries only the binding property of the holotoxin, induces apoptosis in Gb_3Cer/CD77-positive Burkitt lymphoma cell lines. These results suggest that Gb_3Cer/CD77 antigen could transduce the signal leading to apoptosis. Therefore, using a lactate dehydrogenase (LDH) release assay, we examined whether or not TU-1 could induce cytolysis of Daudi cells. As shown in Figure 5, Daudi cells pretreated with TU-1 underwent cytolysis when the cells were added to wells containing immobilized rat anti-mouse IgM antibody. In contrast, the cells pretreated with isotype-matched IgM did not show any cytolysis. These results suggest that TU-1, like VT-B [18], can mimic a signal transduction via Gb_3Cer/CD77 antigen. In addition, as TU-1 was able to induce cytolysis of Daudi cells when cross-linked by plastic-immobilized anti-IgM,

it is likely to be necessary for capping of Gb_3Cer/CD77 antigens by TU-1 to transduce the signal leading to the cytolysis. Furthermore DNA ladder formation during the cytolysis was examined by a DNA fragmentation assay, and evidence of fragmentation was obtained (data not shown). This result therefore suggests that the cell death induced by TU-1 MAb may be apoptosis. Utilization of the TU-1 MAb should provide further information on the mechanism of signal transduction via Gb_3Cer/CD77 antigen leading to apoptosis, and work along this line is in progress.

In this study, we established mouse–mouse hybridoma cells producing monoclonal antibodies directed against Gb_3Cer/CD77 antigen by immunizing mice with Gb_3Cer in an emulsion (RIBI adjuvant) composing monophosphoryl lipid A and trehalose dimycolate. The obtained antibody, TU-1, was immunochemically and histologically characterized, and its epitope was compared with those epitopes of previously described MAbs. TU-1 is a monoclonal antibody directed against Gb_3Cer/CD77 antigen, recognizing the Galα1-4Galβ1-4Glc residue of Gb_3Cer as its epitope, and can thus be utilized as a diagnostic reagent to test for Burkitt's lymphoma among lymphoid cells obtained from patients with lymphoma or leukaemia. Additionally, this MAb can be employed for detection of the blood group P^k antigen in glycolipid extracts of erythrocytes.

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