Production and Characterization of Monoclonal Antibodies against Wheat Flour Diacylgalactosylglycerols

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Seven immunoglobulin M (IgM)-type monoclonal antibodies were developed against wheat diacylgalactosylglycerols (MGDG). That with the highest titer, MG405, was characterized in detail using a solid phase enzyme-linked immunosorbent assay (ELISA). Inhibition experiments with galactose, glycerol, galactosylglycerol, and lipid standards containing fatty acids typical of those in MGDG indicated that MG405 was directed against the whole MGDG molecule rather than parts of its structure. Six other monoclonal antibodies, also IgM type, had similar specificities. Antibody binding was reduced by 30% when fetal bovine serum, used as antibody diluent and blocking agent, was replaced with gelatin in an ELISA carried out with purified MG405. The plasma cofactor β_2-glycoprotein I (β_2GPI) increased binding only slightly, suggesting that some other plasma cofactor may potentiate antibody binding to MGDG. MG405 also appeared not to be an intrinsically low-affinity antibody to β_2GPI. Availability of antibodies such as MG405 could provide a valuable means of investigating polar lipid functionality in foods.

Keywords: *Anti-glycolipid antibody; monoclonal antibody; diacylgalactosylglycerols; MGDG; wheat; bread dough gas retention.*

INTRODUCTION

Nonstarch polar lipids in wheat flour have important effects on gas retention in bread doughs and thus on bread quality (MacRitchie and Gras, 1973; Larsson, 1986). Variation in polar lipid content has been shown for some wheat collections to be an important determinant of the variation in bread making quality that occurs among different cultivars (Békés et al., 1986; Chung et al., 1982). It is likely that the polar lipids, together with proteins dissolved in the aqueous phase of dough, bring about their functional effects by their ability to affect the stability of interfacial films at gas/liquid interfaces in bread doughs during yeast fermentation and the early stages of baking thereby contributing to gas cell stabilization, gas retention, and, hence, bread quality (MacRitchie and Gras, 1973; Larsson, 1986; Gan et al., 1990, 1995). Morphological studies of of gas cell walls in bread dough during fermentation are consistent with that view, and we have proposed a model of dough in which both the gluten/starch bulk phase and the thin liquid lamellae stabilized by surface active molecules in the dough aqueous phase play important roles (Gan et al., 1990, 1995).

Direct experimental proof of our model is lacking, however, largely because of the lack of suitable techniques for investigating problems related to the organization and distribution of lipids in food systems. Monoclonal antibodies (Mab) against wheat polar lipids could provide a valuable means of examining the functional role of such lipids in bread making using immunolocation techniques. Mabs have been developed against wheat and other cereal grain proteins, especially the prolamin storage proteins, in view of their roles as determinants of bread making performance (Skerritt and Underwood, 1986; Dawood et al., 1989; Brett et al., 1990, 1993). No immunological studies of cereal lipids were reported until our recent demonstration that immunogenic responses against wheat galactolipids are elicited readily in mice (Gan et al., 1993, 1994). Here we describe the development of a panel of Mabs against wheat diacylgalactosylglycerols (monogalactosyl diglycerides, MGDG); these have novel specificity characteristics among antipolar lipid Mabs.

MATERIALS AND METHODS

Materials. MGDG, goat anti-mouse IgM (Fab specific) HRP conjugate, rabbit anti-goat IgG HRP conjugate, Freund's adjuvants, phosphate-buffered saline (PBS; pH 7.4), adult bovine serum (ABS), bovine serum albumin (BSA), bovine skin gelatin, and pristane (2,6,10,14-tetramethylpentadecane) were all purchased from Sigma Chemical Co. Ltd. (Dorset, U.K.). Mouse Sp2/0-Ag14 myeloma cell lines, RPMI 1640 tissue culture medium, hypoxanthine–aminopterin–thymidine (HAT; 50×), hypoxanthine–thymidine (HT; 50×), and fetal bovine serum (FBS) were obtained from ICN Flow Biochemicals Ltd. (High Wycombe, U.K.). ImmunoPure MAb isotyping kit I (HRP/ABTS), 1-Step Turbo TMB-ELISA, SuperBlock blocking buffer in PBS, and AbZorb mouse IgM purification kit were supplied by Pierce and Warriner Ltd. (Chester, U.K.). Doma-Driver (hybridoma cell feeder supplement) was obtained from Immune Systems Ltd. (Bristol, U.K.). Poly(ethylene glycol) (PEG) 4000, tris(hydroxymethylamine), and ammonium chloride were provided by BDH Ltd. (Leicester, U.K.). Microtiter plates (Immulon 3) were obtained from Dynatech Laboratories Ltd. (Sussex, U.K.). L-α-Phosphatidylcholine (PC) and L-α-phosphatidylethanolamine (PE) were purchased from Avanti Polar Lipids Inc. (AL). Centricon-100 concentrators were obtained from Amicon Ltd. (Gloucestershire, U.K.). Human plasma β_2-glycoprotein I (β_2GPI) was purchased from Calbiochem-Novabiochem Ltd. (Nottingham, U.K.).

Immunization Scheme. Wheat flour MGDG was used as immunogen; it was shown to be >99% pure by thin layer and high-performance liquid chromatography (results not shown). Six female Balb/c mice (6 weeks old) were immunized by intraperitoneal injection of MGDG using a protocol similar to those used for protein immunogens (Harlow and Lane, 1988). For primary immunization, each mouse was injected with the immunogen (0.1 mg) emulsified in complete Freund's adjuvant (0.25 mL). Each immunized mouse was then boosted with the immunogen (0.05 mg) emulsified in incomplete Freund's adjuvant (0.25 mL) 4 weeks after the primary immunization and at monthly intervals thereafter. Tail bleeds were collected and assessed for antibody titers 10–14 days after each boost

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using the solid phase enzyme-linked immunosorbent assay (ELISA) described below. Four weeks after the second boost and 5 days before fusion, the mouse with the highest antibody titer was injected intraperitoneally with the immunogen (0.05 mg) on 3 consecutive days. Spleen cells were harvested after a further 2 days for hybridoma production.

Hybridoma Production. Hybridomas were produced by PEG-aided (50%, w/v, PEG 4000 in RPMI 1640 medium) fusion of the harvested spleen cells with mouse Sp2/0-Ag14 myeloma cells. The fusion was performed essentially as described previously (Galfre et al., 1977; Liddell and Cryer, 1991). The hybridoma cells were screened for antibody secretion by ELISA (see below) 10−14 days after fusion in order to select cells secreting the desired antibodies. The clones giving antibody binding values 2 times the blank (medium alone/normal mouse serum) values were regarded as positive and expanded into larger wells in 24-well plates so that enough cells could be obtained to give a reliable count prior to cloning. Simultaneously, HAT medium was replaced by HT medium for several passages before returning the cells to the usual RPMI 1640 complete culture medium. Cells from positive wells were cloned by limiting dilution (Liddell and Cryer, 1991) in order to establish a monoclonal cell line.

Propagation of Selected Clones and Production of Antibody in Ascites. Useful amounts of Mab were obtained *in vivo* by intraperitoneal injection of the selected clone into adult mice ($2-9 \times 10^6$ cells/mL in PBS, 0.5 mL/mouse), each of which was primed with pristane (0.5 mL) 3−4 weeks in advance. When the mouse was the size of a normal pregnant female (ca. 2 weeks), ascites fluid was aspirated from the peritoneal cavity. The fluid was then centrifuged at 1500*g* for 10 min and the resulting supernatant stored at −20 °C prior to further characterization.

Development of an ELISA Technique for Anti-MGDG Antibody Detection. The optimum antigen concentration was determined by draughts (checker) board experiments, in which wells were coated with MGDG at various concentrations. The concentration that gave the best discrimination between the Mab and a negative control (10%, v/v, FBS) was selected as optimal and used in subsequent studies. The optimum temperature for antibody binding was determined by incubating coated plates with antibody at either 4, 20, or 37 °C. These temperatures were chosen because they represent the most commonly used assay temperatures in anti-glycolipid antibody (aGL) studies (Marcus et al., 1989; Ariga et al., 1991; Ozawa et al., 1992). The optimum time for primary antibody binding was determined by comparing the binding reactivity of the Mab with its antigen after incubation for different time intervals.

Final ELISA Protocol. Microtiter plates (Immulon 3; Dynatech Laboratories Ltd., Sussex, U.K.) were coated by incubating with the predetermined optimum concentration (31.3 μg/mL of ethanol) of lipid antigen (0.05 mL/well) overnight at 4 °C. All subsequent incubations were carried out at 37 °C for 60 min. The coating solution was removed, and each plate was washed twice with PBS before incubating with 0.1 mL/well of 10% (v/v) FBS in PBS or SuperBlock in PBS for blocking. After washing once with PBS, each well was incubated with an aliquot (0.05 mL) of hybridoma supernatant or ascites fluid diluted appropriately in 10% (v/v) FBS. This was followed by three washes with PBS before incubating 0.05 mL/well of HRP-conjugated goat anti-mouse IgM (Fab specific) and then with 0.05 mL/well of enzyme substrate (1-Step Turbo TMB-ELISA) until the color had developed sufficiently (ca. 5 min). Color development was stopped by adding 1 M H_2SO_4 (0.05 mL) to each well, and the absorbance (A_{450nm}) was measured at 450 nm using a Dynatech MR 5000 plate reader. Unless otherwise stated, the reported absorbance values are those from which the control values for 10% FBS in PBS or Superblock in PBS have been subtracted.

Modified ELISA. To determine the requirement of MG405 for a plasma cofactor for binding to MGDG the standard ELISA protocol was modified to exclude β_2GPI, a plasma protein that has been shown to play an important role in the binding of phospholipids by anti-phospholipid antibodies (aPL). The modified assay was carried out with MG405 that was affinity purified as described below, and 0.3% (w/v) gelatin was used as the antibody diluent and blocking agent. In some experiments, human plasma β_2GPI was added to the gelatin antibody diluent at 5 μg/mL concentration (Roubey et al., 1995) or the purified Mab was preincubated with various concentrations of β_2GPI for 1h before assessing its binding to immobilized MGDG. In other experiments, the β_2GPI was immobilized onto the microtiter plate using a 1.6 μM solution (Keeling et al., 1992) of the protein in PBS (25 μL/well), and the binding of the purified Mab to the immobilized β_2GPI in the absence of MGDG was determined.

Antibody Purification. MG405 was purified using an affinity column containing immobilized goat anti-mouse IgM (AbZorb mouse IgM purification column). MG405 ascites fluid was dialyzed against 1.25 M NaCl, 0.02 M Tris-HCl, pH 7.4, and 0.02% (w/v) sodium azide overnight at 4 °C before being diluted with an equal volume of AbZorb mouse IgM binding buffer. The diluted sample (2 mL) was then applied to the affinity column, which had been equilibrated with the binding buffer. Further binding buffer (3 mL) was added, and the column was allowed to drain. Unbound proteins were removed from the column by extensive washing with binding buffer (ca. 25 mL). The bound IgM was eluted using AbZorb IgM elution buffer; 2 mL fractions were collected and mixed with neutralisation buffer, and the absorbance was measured at 280 nm. The protein fractions were pooled and concentrated using Centricon-100 concentrators to a final concentration of ca. 2.5 mg/mL. The purity of the affinity purified MG405 was checked by SDS−PAGE using 10% gels (Laemmli, 1970). It showed bands characteristic of the heavy and light chains of mouse secretory IgM (M_r 80K and 25K, respectively), whereas no band characteristic of IgG heavy chains (M_r 53K) was observed.

Isotype Determination. Mab isotypes in either culture supernatants or ascites fluids were determined using antigen-mediated ELISA. The assay was performed essentially as described in the final ELISA protocol, except that each well was incubated with goat anti-mouse isotype specific reagents at room temperature for 30 min after incubation with supernatant or ascites fluid. This was followed by 15 min incubation with rabbit anti-goat IgG HRP conjugate and the enzyme substrate.

Antibody Specificity by ELISA Inhibition Assay. MG405 was preincubated with various concentrations of MGDG, galactosylglycerol (GG) (see below), galactose, or a mixture of fatty acid standards representative of those present in wheat galactolipids. The lipids were prepared by sonicating them in FBS, and galactose was dissolved in FBS. The binding activity was then determined in the ELISA. The preincubation was carried out in a microtiter plate that had been blocked previously with 0.3% (w/v) gelatin. Inhibition results are expressed as percentage inhibition of MGDG binding reactivity

$$\% \text{ inhibition} = \frac{A_0 - A_i}{A_0} \times 100 \quad (1)$$

where $A_0 = A_{450nm}$ at zero inhibition and $A_i = A_{450nm}$ at i μg/mL inhibitor.

Preparation and ^{13}C NMR Characterization of Galactosylglycerol. GG, which was unavailable commercially, was prepared by saponifying wheat MGDG (10 mg) in 1 M KOH in 95% ethanol (0.5 mL) overnight at room temperature. Deionized water (0.5 mL) was then added and the pH adjusted to about 6.8 before extraction with diethyl ether (3×0.5 mL) to remove free fatty acids. The aqueous phase was then desalted on a mixed-bed ion exchange column packed with AG501-X8(D) resin. The structure of the product was confirmed by high-resolution ^{13}C NMR spectroscopy using a JEOL (JNM-EX400) FT NMR spectrometer. GG and MGDG were dissolved in D_2O and $CDCl_3$, respectively; tetramethylsilane was used as internal standard. Data were acquired over 30 120.5 Hz into 32 768 data points at operation temperatures of about 23 °C. A DEPT (distortionless enhancement by polarization transfer) NMR spectrum was also obtained to distinguish carbon resonances of the CH_2 and CH groups

Table 1. Binding Reactivities of Monoclonal Anti-MGDG Antibodies to MGDG, and the Inhibition of Binding Reactivity by MGDG, D-(+)-Galactose, Glycerol, GG, and Lipid Standards Containing 16:0, 18:0, 18:1, 18:2, and 18:3 Fatty Acid Chains[a]

			inhibition by various inhibitors (%)				
Mab	isotype	titer	MGDG	GG	glycerol	D-(+)-galactose	lipid standards
MG401	IgM (κ)	1:1500	96	12	5	8	13
MG402	IgM (κ)	1:1600	95	15	3	7	10
MG403	IgM (κ)	1:3200	95	10	5	8	12
MG404	IgM (κ)	1:2000	97	12	2	5	15
MG405	IgM (κ)	1:12 800	99	15	5	9	18
MG406	IgM (κ)	1:2500	96	13	3	6	14
MG407	IgM (κ)	1:3000	97	16	3	5	12

[a] Inhibition experiments were carried out at inhibitor concentration of 0.4 mg/mL, except in the case of MG405, for which they were present at 0.5 mg/mL.

(Sanders and Hunter, 1987). The carbon atom resonances in the NMR spectra (data not shown, available from the authors on request) were assigned by comparison with the chemical shifts given in the literature (Johns et al., 1977; Adebodun et al., 1992), together with those identified by the DEPT experiment. Signals characteristic of those of fatty acid chains were absent from the GG spectrum, which showed only those representing the anomeric carbon atom and galactosyl/glycerol backbone carbon atoms. The NMR results showed that the saponification had been carried out successfully and that the product was indeed GG.

Antibody Cross-Reactivity with Phospholipids. PC and PE preparations from various animal, plant, and microbial sources were coated (31.3 μg/mL) overnight onto microtiter plates. Blocking was performed using 10% (v/v) FBS in the ELISA.

RESULTS

Establishment of Cell Lines Secreting Mabs against MGDG. We have established previously that immune responses were obtainable by intraperitoneal injection of MGDG emulsified in Freund's adjuvant in Balb/c mice (Gan et al., 1993). Antisera diluted 1:100 had binding ratios (antiserum A_{450nm} value divided by the control serum A_{450nm} value) of ca. 9 at an antigen concentration of 31.3 μg/mL. In the research reported here, seven cell lines producing Mabs against wheat MGDG were established by PEG-aided fusion of mouse immune spleen cells and mouse Sp2 myeloma cell lines. All the Mabs produced by these cell lines were determined to be of the IgM class with κ light chains (Table 1). One of the Mabs, designated MG405, was selected for detailed characterization based on its significantly higher binding reactivity with the antigen (Table 1). The titer (half-maximal binding) of MG405 ascites fluid produced *in vivo* using female Balb/c mice was found to be about 1:12 800 using the ELISA protocol developed in this study (see below).

Establishment and Optimization of an ELISA for MGDG. The binding reactivity of MG405 (culture supernatant) to MGDG at varying concentrations of the antigen is shown in Figure 1. The greatest distinction between the binding reactivity of MG405 and that of the negative control was observed at an antigen concentration of about 31.3 μg/mL. This concentration was used subsequently as optimal in the final ELISA protocol. Normal mouse serum produced some slight background color compared with 10% (v/v) FBS alone. It should be noted that in Figure 1 the A_{450nm} values for the 10% FBS controls have not been subtracted from the MG405 binding curves in order to demonstrate the

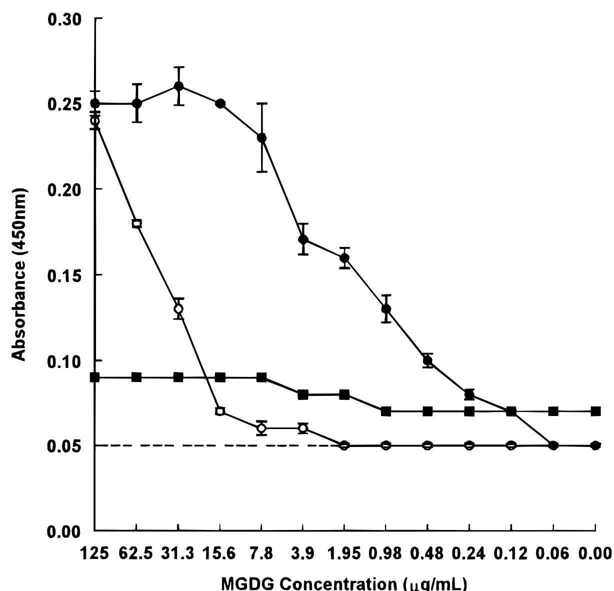

Figure 1. Binding reactivity of MG405 (culture supernatant) to wheat MGDG at various concentrations of the latter. The culture supernatant was diluted 1:20 with either 10% (v/v) FBS (●) or 10% (v/v) FBS containing 0.05% (w/v) Tween 20 (○). Results for normal mouse serum (■) and 10% (v/v) FBS control (dotted line) are also shown. Note that these assays were carried out at 37 °C and that the absorbance values for the 10% FBS controls have not been subtracted from the MG405 results either with or without Tween 20 in order to show the level of the background absorbances obtained. The error bars indicate the standard deviations of replicates.

level of background in the ELISA. A_{450nm} values presented in other figures and tables are values from which the A_{450nm} values of the controls have been subtracted. It should also be noted that the assays, the results of which are presented in Figure 1, were carried out at 37 °C. The absorbance range obtained in the ELISA was very narrow compared with ranges normally observed with antigens, such as proteins and peptides. Despite this, the precision of the results obtained in the ELISA was very good as indicated by the low standard deviations of the replicate values (see Figures 1 and 2).

The use of 0.05% (w/v) Tween 20 in antibody diluents has been reported to enhance IgM aPL binding (Cheng, 1988; Cheng and Yap, 1988; Ravindranath et al., 1994). In contrast, we observed that it reduced the binding reactivity of our Mab substantially, with no antibody binding being detected at antigen concentrations of about 15.6 μg/mL and below (Figure1). This was probably a result of the lipid antigen being washed away by the detergent, a widely reported observation in antilipid antibody immunoassays (Young et al., 1981; Gharavi and Lockshin, 1988; Harris, 1990; Harris et al., 1987; Cabral et al., 1994).

Incubation at 37 °C enhanced antibody binding considerably compared with incubations carried out at lower temperatures (Figure 2). The length of the incubation time of the Mab with MGDG had no appreciable effect on its binding reactivity. A 1 h incubation was sufficient for antibody binding ($A_{450nm,1h}$ = 0.220): longer incubation periods did not enhance the binding significantly ($A_{450nm,2h}$ = 0.221; $A_{450nm,3h}$ = 0.223). All buffers tested for blocking in the ELISA [1% (w/v) BSA, 0.3% (w/v) gelatin, 10% (v/v) ABS, 10% (v/v) FBS, and SuperBlock in PBS] gave similar backgrounds. Freshly made 10% (v/v) FBS and SuperBlock gave slightly better blocking, however (results not shown). The procedure we have adopted, therefore, involves the

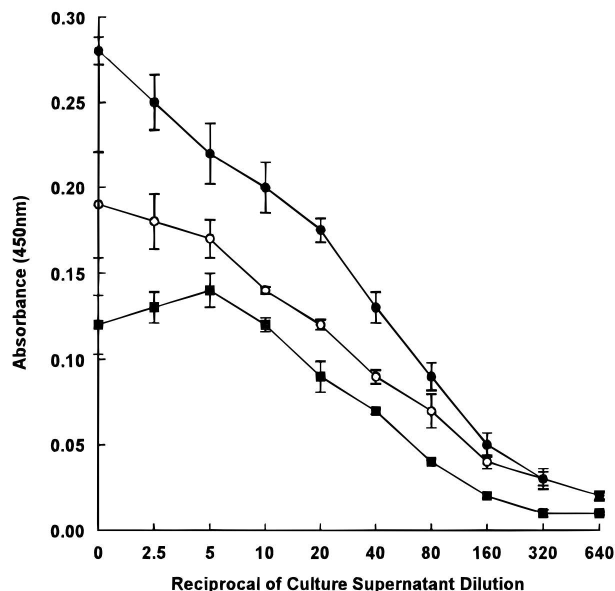

Figure 2. Binding reactivity of serial dilutions of MG405 culture supernatant to MGDG at (●) 37 °C, (○) 20°C, and (■) 4 °C. The error bars indicate the standard deviations of replicates.

Table 2. Binding Reactivities toward MGDG and β_2GPI of MG405 Diluted in either 0.3% (w/v) Gelatin or 10% (v/v) FBS and the Effect of Adding β2GPI to the Gelatin Diluent[a]

antibody	antibody diluent	immobilized antigen MGDG	immobilized antigen β_2GPI
purified MG405	0.3% gelatin	0.161 ± 0.007	0.003 ± 0.001
purified MG405	0.3% gelatin + β_2GPI[b]	0.187 ± 0.008	
purified MG405	10% FBS	0.230 ± 0.001	
MG405	10% FBS	0.234 ± 0.001	0.008 ± 0.001

[a] Values quoted are A_{450nm} in the ELISA. [b] Added at 5 μg/mL.

use of PBS alone for washing, 10% (v/v) FBS for antibody dilution, 10% (v/v) FBS or SuperBlock for blocking and 1 h incubation at 37 °C for anti-MGDG IgM antibody detection and characterization.

Investigations of the Possible Role of β_2GPI. When assessed in the modified ELISA using 0.3% (w/v) gelatin as antibody diluent and blocking agent to exclude any cofactor that may be present in bovine serum, the binding reactivity of the affinity-purified MG405 was about 30% lower than that of the same Mab diluted in 10% (v/v) FBS (Table 2). The addition of human plasma β_2GPI to the 0.3% (w/v) gelatin diluent in the same assay produced only a small increase in antibody binding. This indicates that β_2GPI is not an absolute requirement for the anti-MGDG antibody binding. Other factors may be responsible for the reduction in binding reactivity when 10% (v/v) FBS was replaced with 0.3% gelatin in the modified ELISA, but they have not been characterized.

Neither MG405 diluted in 10% (v/v) FBS nor affinity-purified MG405 diluted in 0.3% (w/v) gelatin recognized immobilized β_2GPI on the ELISA plate (Table 2). In another experiment, in which the purified Mab was preincubated with β_2GPI before assessing its binding to immobilized MGDG, no inhibition of antibody binding to MGDG was observed over a β_2GPI concentration range of 0−100 μg/mL (results not shown). These results indicate that MG405 is not an intrinsically low-affinity antibody to β_2GPI.

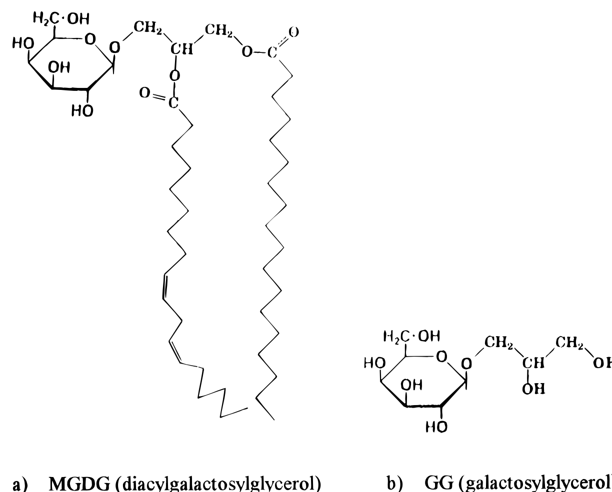

Figure 3. Structures of (a) diacylgalactosylglycerol (MGDG) and (b) galactosylglycerol (GG).

Antigenic Specificity of the Anti-MGDG Mab. The nature of the epitope recognized by MG405 in the ELISA was investigated in inhibition experiments. Preincubation of the Mab with lipid standards containing 16:0, 18:0, 18:1, 18:2, and 18:3 fatty acids typical of those present in wheat MGDG (Figure 3a) produced only a small degree (15-18%) of inhibition even at high concentrations (Figure 4), suggesting that these fatty acid chains are not major determinants for antigen recognition by the anti-MGDG Mab. Furthermore, neither galactose nor glycerol was observed to inhibit the binding between MG405 and MGDG (Figure 4). To test whether the galactosyl/glycerol backbone might have been the epitope against which MG405 was directed, an additional inhibition experiment was carried out using GG (Figure 3b), which was prepared by saponifying MGDG and the structure of which was verified by NMR. GG produced only a small degree (10−15%) of inhibition. The inhibition results thus suggested that neither the fatty acid chains nor the carbohydrate moiety of the MGDG molecule was a critical epitope for MG405 recognition. Addition of MGDG, on the other hand, inhibited the binding reactivity of MG405 considerably and gave almost total inhibition of antibody binding at high concentrations (Figure 4). This result suggests that the antigenic determinant of the Mab in the ELISA was the complete MGDG molecule. Mabs produced by the other six cell lines had similar specificity characteristics (Table 1).

Assessment of Cross-Reactivity with Phospholipids. The reactivity of the MG405 antibody toward phospholipids was also determined. Cross-reactivity was assessed using PC and PE, which represent the major classes of wheat phospholipids. Purified wheat phospholipid standards were not available, and thus, purified phospholipids from a variety of animal, plant, and microbial sources were used. No binding reactivity with these lipids was detected irrespective of their origin (Table 3).

DISCUSSION

Lipids are generally reputed to be only weakly or nonimmunogenic, and obtaining immune responses against glycolipid antigens has been reported to pose considerable difficulties (Brodin et al., 1986). The preferred methods for production of aGL are by immunization either with purified glycolipids adsorbed

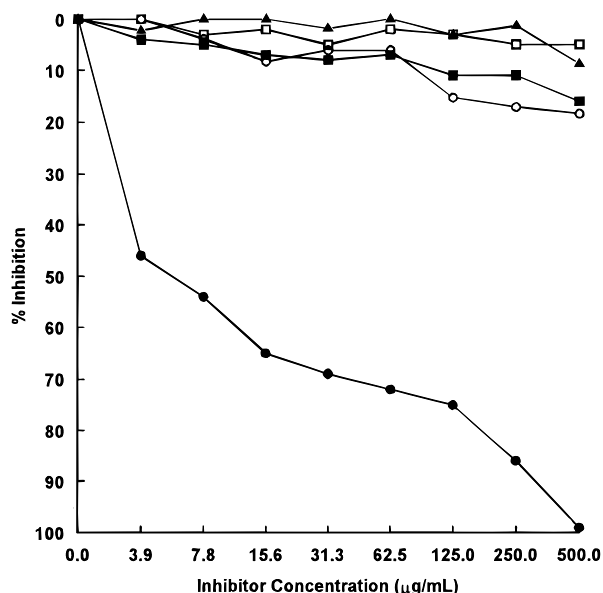

Figure 4. Inhibition of MG405 (ascites fluid diluted 1:20 000) binding to MGDG by varying concentrations of MGDG (●), GG (■), D-(+)galactose (▲), glycerol (□), and lipid standards containing 16:0, 18:0, 18:1, 18:2, and 18:3 fatty acid chains (○).

Table 3. Antibody Binding Reactivity against PC and PE from Animal (Egg), Plant (Soya), and Microbial (*E. coli*) Sources

immobilized lipid	A_{450nm}	immobilized lipid	A_{450nm}
MGDG	0.229 ± 0.001	PE (*E. coli*)	0.002 ± 0.001
PC (egg)	0.006 ± 0.001	PE (soya)	0.000 ± 0.000
PC (soya)	0.002 ± 0.001		

onto the surfaces of acid-treated *Salmonella minnesota* cells (Young et al., 1981; Ozawa et al., 1992) or with purified glycolipids incorporated into artificial lipid vesicles (liposomes) (Galanos et al., 1971; Alving et al., 1980; Brodin et al., 1986). Immunization with adjuvant mixture containing keyhole limpet hemocyanin and supplemental *Mycobacterium tuberculosis* was also adapted successfully in producing antibodies against galactocerebroside (Benjamins et al., 1987).

In contrast, we have shown here that high-titer Mabs against wheat flour MGDG can be produced by direct immunization without the need for a bacterial cell carrier or incorporation of the antigen into liposomes. Mabs that reacted with MGDG were produced previously by immunization with spinach thylakoid lipid membranes, the main components of which are galactolipids similar to those present in wheat flour (Gounaris et al., 1984). The Mabs were not characterized in detail, however, and the clones are no longer available (K. Gounaris, personal communication).

aGL have generally been found to have low titer and affinity compared with anti-peptide antibodies. This low affinity results in a narrower range of absorbance values in ELISAs for glycolipids than is normally found for antigens, such as proteins. Thus, absorbance ranges reported for ELISAs for glycolipids are typically in the range 0−0.4/0.5 (Taki et al., 1984, 1987; Ozawa et al., 1992), whereas absorbance ranges obtained in ELISAs for protein antigens are generally in the range 0−1.5/2.0. Apart from the low affinity of aGL, factors, such as antibody isotype, the type of ELISA plate, and the types of enzyme conjugate and substrate used, can affect the fine sensitivity of aGL in ELISAs (Marcus et al., 1989; Kurby, 1994; Ravindranath et al., 1994). The fact that our Mabs were of the IgM type, which often have lower affinity than IgG-type antibodies (Kurby, 1994), may explain the low absorbance maxima we obtained, even by aGL ELISA standards. However, our primary concern is with antibody specificity rather than affinity. Despite the very low absorbance range we obtained in the ELISAs, the precision of the assays was good as indicated by the low standard deviations obtained for replicates across the range of MGDG concentrations studied.

The greater reactivity observed for antigen−antibody binding at 37 °C than at lower temperatures suggests that antigen recognition by MG405 is an endothermic event. Early observations with aPL led to proposals that temperature-dependent increases in binding reactivity may be due to thermally induced phase transitions in the lipid antigen (Rauch and Janoff, 1990; Rauch et al., 1986). The temperature-induced changes in antibody binding could also be related to thermally induced changes in the antigen binding site. This could involve temperature-induced changes in the balance between hydrophobic interactions (which would be favored at higher temperatures) and polar/ionic bonds (which would be favored at lower temperatures). Such effects may be especially important in the case of lipid antigens. Different types of antibodies may also show different responses to temperature changes. Thus, IgM-type antibodies were reported to fix complement most efficiently at 37 °C and IgG type most efficiently at 4 °C (Stollar and Sandberg, 1966; Sandberg and Stollar, 1966). Lockshin et al. (1988), however, found that the binding of IgG-type aPL was temperature dependent but not that of the IgM type. This contrasts with the results obtained here with MG405, which is of the IgM isotype, but its MGDG binding reactivity was found to be temperature dependent.

It is well established also that the use of bovine serum-based diluents and blocking solutions greatly improves the discrimination between positive and negative samples for anti-cardiolipin antibodies (Loizou et al., 1985; Gharavi et al., 1987), and it has been reported that some aPL bind to anionic phospholipids only in the presence of a serum cofactor, β_2GPI (McNeil et al., 1990; Galli et al., 1990; Matsuura et al., 1990; Chamley et al., 1991; Roubey et al., 1992; Jones et al., 1992). We have not discovered any report, however, of the involvement of β_2GPI in the antigenic specificity of aGL.

Our present data showed that, although inclusion of β_2GPI at 5 μg/mL enhanced the binding of MG405 to MGDG, the degree of enhancement was small, suggesting that a factor other than β_2GPI may have been responsible for the somewhat reduced binding reactivity when FBS was replaced by gelatin in the modified ELISA. FBS is a complex mixture of proteins, among which may be various lipid-binding proteins, including serum albumin. Such lipid-binding proteins may play a role in the somewhat reduced binding of MG405 to MGDG when FBS was replaced by gelatin in the modified ELISA. MG405 did not recognize β_2GPI immobilized on ELISA plates, and neither did its preincubation with β_2GPI inhibit its binding to immobilized MGDG. Thus, MG405 appears not to be an intrinsically low-affinity antibody to β_2GPI, unlike aPL.

Antibodies against complex glycoconjugates (both glycolipids and glycoproteins) appear to be directed primarily to the carbohydrate moieties of their antigens (Young et al., 1981; Benjamins et al., 1987; Watkins,

1991). In the case of aPL, however, the lipid moieties, such as the C18 fatty acid chains of phospholipids, have been reported to be critical epitopes (Levy et al., 1990), and such antibodies also share the common characteristic of having a "subsite" in the antibody combining site that has specificity for phosphate esters (Alving, 1986; Levy et al., 1990). Our inhibition data have shown that the antigenicity of MGDG was not determined by the galactose residue or the galactosylglycerol moiety, and neither did the lipid moieties appear to be critical epitopes. The complete inhibition produced by MGDG suggested that MG405 and the other Mabs were directed against the whole MGDG molecule and not their carbohydrate or lipid moieties.

In summary, this research has resulted in the development of a high-titer Mab against MGDG, which has unique specificity characteristics among antipolar lipid antibodies in that the epitope against which the antibody was directed appeared to be the whole molecule rather than the carbohydrate or fatty acid moiety. Other Mabs produced against MGDG also appeared to have similar specificity characteristics. The Mab was produced by direct immunization without the need for adsorption of the immunogen onto acid-treated bacterial cells or incorporation into liposomes, as has been recommended in other work. Further investigation is needed to identify a serum cofactor that may be involved in enhancing the binding of the MG405. This cofactor is not β_2GPI, which has been found to be an obligatory cofactor in the binding of aPLs and phospholipids. The Mab is potentially valuable for immunohistochemical investigations of the functionality of galactolipids in food systems and in studies of plant membrane structure and function in general.

ABBREVIATIONS USED

β_2GPI, β_2-glycoprotein I; BSA, bovine serum albumin; DEPT, distortionless enhancement by polarization transfer; FBS, fetal bovine serum; GG, galactosylglycerol; MGDG, diacylgalactosylglycerol (monogalactosyl diglyceride); DMSO, dimethyl sulfoxide; aGL, anti-glycolipid antibodies; aPL, anti-phospholipid antibodies; ELISA, enzyme-linked immunosorbent assay; NMR, nuclear magnetic resonance; HAT, hypoxanthine–aminopterin–thymidine; HT, hypoxanthine–thymidine; HRP, horseradish peroxidase; Ig, immunoglobulin; Mab, monoclonal antibody; A_{450nm}, absorbance at 450 nm; PBS, phosphate-buffered saline; PEG, poly(ethylene glycol); TMB, 3,3′,5,5′-tetramethylbenzidine; PC, L-α-phosphatidylcholine; PE, L-α-phosphatidylethanolamine.

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