Monoclonal antibodies recognizing epitopes carried on both glycolipids and glycoproteins of the human milk fat globule membrane

R. A. Jeffrey MCILHINNEY, Shashikant PATEL and Martin E. GORE

Ludwig Institute for Cancer Research, The Royal Marsden Hospital, Sutton, Surrey SM2 5PX, U.K.

(Received 13 September 1984/Accepted 30 November 1984)

The molecules of the human milk fat globule membrane (MFGM) which bind four murine monoclonal antibodies (LICR LON M3, M8, M18 and M24) raised against the human MFGM have been identified. By using 'Western' blotting [Burnette (1981) *Anal. Biochem.* **112**, 195–203] it was shown that each antibody reacted with a different set of proteins. M3 and M24 were similar in their pattern of reaction with the membrane proteins, but were quite distinct from M8 and M18, which also differed from each other. Glycopeptides prepared from the MFGM by exhaustive Pronase digestion were able to inhibit partially the binding of M3 and M24, and prevent totally the binding of M8 and M18, to the MFGM in an enzyme-linked immunoabsorbent assay. Oligosaccharides obtained by the deproteination of human milk also completely inhibited the binding of M3, M18 and M24 to the MFGM. However, the binding of M8 was not inhibited by these saccharides, and therefore M8 may not be recognizing a simple carbohydrate determinant. By using an enzyme-linked assay, M8 and M18 were shown not to bind to MFGM glycolipid, whereas M3 and M24 did, and this was confirmed by overlaying thin layer chromatograms of MFGM lipids with these antibodies. Both M3 and M24 showed a similar complex pattern of reaction, binding to more than one glycolipid moiety. By these means all four antibodies have been shown to react with antigens which involve carbohydrate side chains carried on different proteins, and two were also shown to react with such determinants on glycolipids.

Lipid is secreted by the mammary gland in the form of droplets surrounded by a membrane known as the milk fat globule membrane (MFGM). The apical plasma membrane of the lactating cell envelopes the lipid droplet to form the MFGM (Saacke & Heald, 1974; Bargmann & Knoop, 1959). The MFGM provides a convenient source of purified breast epithelial cell membrane since it is readily extracted in large quantities from human milk. Consequently, it has been used both as an immunogen in the production of antisera to human breast epithelial antigens (Ceriani *et al.*, 1977; Heyderman *et al.*, 1979; Sloane & Ormerod, 1981) and in the purification of molecules of the human MFGM (Franke *et al.*, 1981; Freudenstein *et al.*, 1979; Imam *et al.*, 1981).

Abbreviations used: SDS, sodium dodecyl sulphate; MFGM, milk fat globule membrane; e.i.s.a., enzyme-linked immunoabsorbent assay; EMA, epithelial membrane antigen.

More recently a number of groups have produced monoclonal antibodies (Köhler & Milstein, 1975) to MFGM (Foster *et al.*, 1982a; Taylor-Papadimitriou *et al.*, 1981; Hilken *et al.*, 1982), in order to produce reagents to study normal and neoplastic breast tissue. To date none of these antibodies has been breast-specific, although all have shown interesting patterns of reactivity with normal and malignant epithelial cells from both breast and other tissues (Foster *et al.*, 1982b; Arklie *et al.*, 1981). An interesting aspect of these patterns has been the heterogeneous manner in which the antibodies bind to cells both in tissue sections and in culture. For example, in breast sections there was variability in staining between adjacent cells as well as between adjacent areas of the same breast (Foster *et al.*, 1982a; Hilken *et al.*, 1982; Arklie *et al.*, 1981). Similar patterns of heterogeneity have been described in tumour sections (Foster *et al.*, 1982b; Arklie *et al.*, 1981) and in viable intact epithelial sheets obtained from

normal human breast (Edwards & Brooks, 1984). There have been many explanations put forward to explain this heterogeneity. These include the existence of subsets of epithelial cells (Edwards, 1983) or different degrees of differentiation within a tissue (Foster *et al.*, 1982a; Arklie *et al.*, 1981). However, the nature of this epithelial phenotypic variation is difficult to explain without evidence as to the types of molecules reacting with these antibodies. Some breast monoclonal antibodies have been shown to recognize complex carbohydrate epitopes (Burchell *et al.*, 1983) but many have not been fully characterized (Hilkens *et al.*, 1982).

Four monoclonal antibodies (LICR LON M3, M8, M18 and M24) raised at this Institute against human MFGM demonstrate a heterogeneous staining pattern on epithelia (Foster *et al.*, 1982a; Edwards & Brooks, 1984) and this report describes the characterization of their antigens. All four antibodies react with carbohydrate determinants carried on proteins of the MFGM and two (M3 and M24) also react with glycolipids of the MFGM. These results are compared with those obtained with other monoclonal antibodies raised against human MFGM and the phenomenon of heterogeneity is discussed in the light of this comparison.

Materials and methods

Materials

The production of the monoclonal antibodies LICR LON M3, M18, M24 and M18 and detailed descriptions of their tissue specificities have been described previously (Foster *et al.*, 1982a,b). In all experiments supernatants from cultures of the hybridoma cells were used at appropriate dilutions. The culture medium used was 5% CO₂-buffered Dulbecco's modified Eagles' medium containing 10% (v/v) foetal calf serum and 100 µg of kanamycin/ml. Culture medium was harvested, centrifuged at 3000g_{av.} for 15 min and frozen in aliquots. It was thawed once before use. M8 belongs to the IgG1 subclass of immunoglobulin and M3, M18 and M24 are all IgM antibodies (Foster *et al.*, 1982a,b).

CSL urease-conjugated rabbit anti-[mouse (Fab)₂]immunoglobulin and CSL urease substrate for the e.l.i.s.a. assay were purchased from Sera Labs.

Polystyrene and polyvinylchloride 96-well microtitre plates were purchased from Dynatech. Glycolipid standards were supplied by Supelco and Pronase was obtained from Boehringer. All other chemicals unless otherwise specified were obtained from Sigma. HPTLC plates (silica gel 60) were purchased from Merck.

Preparation of MFGM

Milk from mothers who had delivered 1–7 days earlier was collected, pooled and frozen at –20°C until processed. MFGM was separated from the milk by freeze-thawing and homogenization and recovered by high-speed centrifugation as previously described (Gore *et al.*, 1984).

Lipid extractions

A total lipid extract was prepared from MFGM equivalent to 76 mg of membrane protein by the method of Esselman *et al.* (1972), yielding 42.9 mg of material (by weight). The lipid was stored at –20°C under N₂.

Further purification of these lipids, by Folch partition and silicic acid chromatography, was performed as described by Esselman *et al.* (1972).

A sample of the Folch upper phase lipids was separated into charged and uncharged lipids by chromatography on DEAE-Sephadex A-25 according to the method of Yu & Ledeen (1972).

Pronase digestion

The protein pellet obtained after extraction of the MFGM lipids was exhaustively digested at 37°C for 24 h with Pronase (2.6 mg) in 5 ml of 50 mM-Tris/HCl (pH 8.0) containing 150 mM-NaCl and 1.5 mM-MgCl₂. A further 2.6 mg of Pronase was then added and the digestion continued for an additional 24 h. The digested material was clarified by centrifugation (10000g_{av.}, 20 min) and the supernatant was freeze-dried. The glycopeptides were fractionated on a Sephadex G-50 column in ammonium acetate buffer (0.1 M, pH 4.5) and the resulting fractions were analysed by the phenol/H₂SO₄ method (Ashwell, 1966) in order to determine their sugar content. The two major peaks of sugar-containing material (peaks 1 and 2) obtained were freeze-dried, yielding 6.3 mg and 9.0 mg of glycopeptides (by weight) respectively.

Preparation of milk oligosaccharides

Oligosaccharides were prepared from 360 ml of milk as described by Smith *et al.* (1978). In our hands, the unretarded saccharides still contained some sialic acid after DEAE-cellulose chromatography (see Table 1) and it was this fraction which contained the major antigenic activity of the starting material. No protein could be detected in these saccharide samples (8 mg by weight) as determined by the Bio-Rad dye-binding protein assay, with a sensitivity of 1 µg. Further fractionation of these saccharides was obtained by chromatography on Bio-Gel P-2 in distilled water with phenol/H₂SO₄ (Ashwell, 1966) to detect the sugar-containing peaks. The sugar composition of these peaks was determined by the method of Clamp *et al.* (1971).

using a Perkin-Elmer F30 gas chromatograph with D-mannitol as an internal standard. These determinations indicated that carbohydrate accounted for >90% of the dry weight of the separated saccharides.

Micro-e.l.i.s.a. assays

These were performed using MFGM-coated 96-well polystyrene microtitre plates as described in Gore *et al.* (1984). Micro-e.l.i.s.a. assays involving lipid were performed using polyvinylchloride microtitre plates coated with lipid dissolved in ethanol as described by Magnani *et al.* (1982).

The concentration of lipid and sugar in the samples varied for each assay and will therefore be indicated in the appropriate Figure legends. In the competition assays the antibody supernatant was used at a dilution that gave 80% of maximum binding, usually 1:20 in phosphate-buffered saline containing bovine serum albumin. The antibodies were used at a saturating dilution, usually 1:1 in phosphate-buffered saline containing bovine serum albumin, for all other assays.

Protein estimations

Unless otherwise stated all protein determinations were performed by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

SDS/polyacrylamide-gel electrophoresis

MFGM proteins were separated in SDS/10% polyacrylamide gels using the discontinuous gel system described by Laemmli (1970); after separation the proteins were electrophoretically transferred to nitrocellulose sheets. Antibody overlays of the nitrocellulose sheets were performed as described by Burnette (1981). Autoradiography was performed using Kodak XS-1 X-ray film with Ilford Fast Tungstate intensifying screens at -40°C.

Table 1. Sugar composition of the Bio-Gel-fractionated saccharides

Abbreviation used: N.D., not detected.

Sugar	Carbohydrate content (mmol/mg)			
	Peak 1	Peak 2	Peak 3	Peak 4
Fucose	734.3	733.1	510.6	92.30
Mannose	N.D.	813.9	N.D.	N.D.
Galactose	2522.4	1158.2	1531.2	1759.7
Glucose	371.8	1038.2	682.6	4953.9
N-Acetylgalactos- amine	484.2	219.3	116.2	N.D.
N-Acetylglucosamine	1683.8	1206.1	1033.3	11.4
N-Acetylneuraminic acid	657.0	386.3	645.2	N.D.

Lipid t.l.c.

Lipids were separated on high-performance t.l.c. (HPTLC) plates with a chloroform/methanol/water (60:30:7, by vol.) solvent system. Antibody overlays of these plates were performed as described by Magnani *et al.* (1982).

Results

Identification of the MFGM proteins recognized by the antibodies M3, M8, M18 and M24

The proteins of the human MFGM that bind the antibodies were identified by immunoblotting strips of nitrocellulose to which separated MFGM proteins had been transferred (Burnette, 1981). The results (Fig. 1) showed that each antibody had a distinctive pattern of binding and M3, M18 and M24 reacted with more than one protein band. M8 bound to a single band of high M_r , which did not enter the separating gel. M18 also bound strongly to a polydisperse high- M_r material, but in addition reacted with proteins of 65 kDa and 40 kDa. M3 and M24 had a similar pattern of binding to proteins of 93 kDa, 50 kDa and 42 kDa, but M24 also

Fig. 1. 'Western' blotting of human MFGM proteins using M3 (lane g), M8 (lane d), M18 (lane c) and M24 (lanes a and f)

MFGM proteins were separated on a preparative SDS/polyacrylamide gel and transferred to nitrocellulose paper as described in the Materials and methods section. The strips of nitrocellulose were overlaid with the appropriate antibody supernatant. Lane (b) serves as a control for specificity since it was overlaid with an antibody which did not react with the MFGM. Lane (e) was overlaid with an anti-MFGM antibody arising from a separate fusion and is not discussed further.

bound to a pair of high- M_r bands and with bands of 72 and 70 kDa.

The simplest explanation for these complex patterns of reaction is that the antibodies recognize carbohydrate determinants which are carried on more than one glycoprotein. In order to test this hypothesis we attempted to inhibit competitively the binding of these antibodies to the MFGM by glycopeptides prepared from MFGM proteins and oligosaccharides prepared from human milk.

Inhibition studies using glycopeptides

The glycopeptides were prepared from delipidated MFGM by exhaustive Pronase digestion, fractionated on Sephadex G-50, and two carbohydrate peaks were obtained and pooled (Fig. 2). Peak 1 probably comprised partially digested MFGM proteins, glycopeptides with extensive carbohydrate side chains and Pronase-resistant mucin-like MFGM molecules such as PAS-0 (Shimizu & Yamauchi, 1982). Peak 2 comprised the fully digested glycopeptides (Finne & Krusius, 1982). Both fractions were tested for their ability to inhibit the binding of each of the antibodies to the MFGM, using MFGM adsorbed onto a plastic microtitre dish in the micro-e.l.i.s.a. assay.

M8 and M18 were completely inhibited from binding to the MFGM in the presence of glycopeptides from either peak 1 or peak 2. However, at the highest concentration of glycopeptide available for testing peak 1 could only partially (40%) inhibit the binding of M3 and M24 and peak-2 glycopeptides had no effect. The simplest explanation for the partial inhibition obtained with peak-1 glycopeptides is that there was insufficient material

present to abolish completely the binding of M3 and M24 to the MFGM. These results clearly indicated that M8 and M18 recognize MFGM epitopes carried on glycopeptides but were equivocal for M3 and M24. In order to determine whether these two antibodies were binding carbohydrate determinants, the ability of human milk oligosaccharides to inhibit competitively their binding to the human MFGM was studied.

The milk oligosaccharides were capable of completely inhibiting the binding to MFGM of all the antibodies. However, following BaSO_4 precipitation M3, M18 and M24 could still be inhibited by these sugars whereas M8 could not. The results of this experiment and those described in the previous section suggest that M8 is not reacting with a simple sugar residue but with a more complex epitope involving a carbohydrate side chain and/or the protein sequence to which it is attached.

The saccharides were fractionated further by size exclusion chromatography in Bio-Gel P-2, the fractions making up the included volume of this column were pooled as shown in Fig. 3, and the sugar composition of each peak is indicated in Table 1. Samples from each peak were tested at different dilutions for their ability to inhibit the binding of M3, M24 and M18. M8 was not included in this study for the reasons given above. The results (Fig. 4) showed that M18 was only inhibited by peak 4 which contained small saccharides, predominantly lactose (Table 1), whereas the larger saccharides of peak 2, which interestingly was the only peak to contain mannose (see Table 1), contained the major competitive activity to M3 and M24. The small amount of M3- and

Fig. 2. Chromatography of human MFGM glycopeptides on Sephadex G-50

The Pronase-digested MFGM glycopeptides were prepared as described in the Materials and methods section, and loaded onto a column of Sephadex G-50 (20 cm $\times$ 1.6 cm) equilibrated with ammonium acetate (0.1 M, pH 4.5). Fractions (2.3 ml) were collected, and the carbohydrate content of 100 μ l aliquots was determined with the phenol/ H_2SO_4 method. The fractions were pooled as indicated.

Fig. 3. Fractionation of milk sugars

Deproteinized milk sugars (400 mg) were subjected to chromatography on a Bio-Gel P-2 (166 ml) column in distilled water. Fractions (2.1 ml) were collected and the carbohydrate content of aliquots (100 μ l) of these determined with the phenol/ H_2SO_4 method. The fractions were pooled as shown and freeze-dried.

Fig. 4. Inhibition of the binding of M3 (a), M24 (b) and M18 (c) to human MFGM by fractionated milk sugars. Milk sugars separated on Bio-Gel P-2 (see Fig. 3) were used to prevent the binding of the antibodies to MFGM in an e.i.s.a. assay. Sugars from each peak were used at the concentrations shown. The sugars used were peak 3 (●), peak 1 (■), peak 2 (▲) and peak 4 (□); 20 μ l of the concentration indicated was added to each well. All points are the means of triplicate determinations, and the s.d. on all points was less than 10%. In (c) the lower scale refers only to the concentration of peak-4 sugars.

M24-reactive material found in both peaks 1 and 3 could be due to contamination from peak 2. Peak 4 did not inhibit M3 and M24.

These observations support the hypothesis that the antibodies are all reacting with determinants which contain carbohydrate, although M8 clearly is not reacting with a simple sugar specificity. They also show that M18 is clearly distinct from M3 and M24 in its reaction with a lower- M_r saccharide, and suggest that, although M3 and M24 may see different sugar side chains, these are of a similar size.

Investigations of MFGM lipid binding

When the upper and the lower phase MFGM lipids were tested for their ability to bind the four antibodies, using the micro-e.i.s.a. assay previously described, it was found that M3 and M24 reacted with lipids derived from both phases, the

upper aqueous Folch fraction and the lower more hydrophobic Folch fraction. M8 and M18 did not however, bind to either phase. Similarly no reaction with MFGM-derived lipids was found with either the monoclonal antibody SSEA-1 (Solter & Knowles, 1978) or a polyclonal rabbit anti-(Forssman antigen) serum (results not shown). Quantitative binding studies were performed by dilution of the upper and lower phase lipids. These assays showed that by weight the upper-phase lipids contained 30 times more antigenic activity for both M3 and M24 than did the lower phase lipids.

The ability of M3 and M24 to bind lipids was also tested by subjecting both upper and lower Folch fractions to t.l.c. then incubating the chromatograms with both antibodies separately (Magnani *et al.*, 1982). Autoradiography of the resultant chromatogram revealed that both the upper and the lower Folch fractions contained glycolipids which bound M3 and M24 (Fig. 5). Similar experiments also confirmed that M8 and M18 did not react with MFGM glycolipids (results not shown). M3 and M24 react with several glycolipids in the upper phase, but with only one band in the lower phase and then rather weakly. This lower phase glycolipid band exhibits the same mobility as the major M3- and M24-binding upper phase band (Fig. 5). The two bands may therefore be the same glycolipid and its presence in the lower phase may represent that part of it which failed to partition into the upper phase during the Folch extraction. M24 reacted strongly with a glycolipid band which consistently ran slightly slower than that which bound M3. The other glycolipid bands which reacted with both antibodies showed similar differences in their relative mobilities. None of the major M3 or M24 reactive bands co-migrated with the major glycolipid bands in the MFGM lipid preparations (Fig. 5b). This suggests that the antibodies are reacting with minor components of these preparations.

Discussion

This study was undertaken to identify those molecules in the human MFGM which react with the four monoclonal antibodies M3, M8, M18 and M24. We have shown that all of these antibodies bind determinants which involve carbohydrate groups; these determinants are carried on MFGM glycoproteins and, in the case of M3 and M24, on MFGM glycolipids as well. The antibodies M3, M18 and M24 appear to react solely with the carbohydrate moieties carried on these molecules since their binding to the MFGM can be inhibited by milk oligosaccharides. This conclusion has been confirmed in the case of the epitope bound by M18, by recent reports which identified this epitope as

Fig. 5. T.l.c. of MFGM glycolipids

(a) T.l.c. on silica gel of upper (A, C) and lower phase (B, D) MFGM glycolipids followed by immunoblotting with M3 (C, D) or M24 (A, B). The lipids binding M3 and M24 were detected as described in the Materials and methods section. (b) A parallel plate loaded with 15 µg of upper phase (UP) and lower phase (LP) MFGM lipids was stained with diphenylamine/aniline (Esselman *et al.*, 1972) to identify the glycolipid bands. A tracing from this plate is shown. Minor components are shown with light shading. Abbreviations used: or, origin; CDH, ceramide dihexosides; CTH, ceramide trihexosides; Globo, globoside; G_{M1} , G_{D1} , G_{T1} , gangliosides (Svennerholm, 1963).

the carbohydrate sequence $\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow$ (Foster, 1983; Gooi *et al.*, 1983). Full characterization of the epitopes bound by M3 and M24 has however proved difficult but it seems that neither of these antibodies reacts with the Y- or the Forsmann antigen, red blood cells from groups A, B or O, or with red blood cells carrying the Lewis antigens $\text{Lewis}^a b^-$, $\text{Lewis}^a b^+$, $\text{Lewis}^a b^-$ (R. A. J. McIlhinney, unpublished work). The epitope bound by M3 and M24 also do not seem to be that recognized by the monoclonal antibody SSEA-1, $\text{Gal}\beta 1 \rightarrow 4(\text{Fuc}\alpha 1 \rightarrow 3)\text{GlcNAc}\beta 1 \rightarrow 6$ (Gooi *et al.*, 1981), firstly because both the former antibodies bind MFGM-derived glycolipids whereas SSEA-1 does not, and secondly because a synthetic oligosaccharide with the structure shown above failed to inhibit M3 and M24 binding to the MFGM (R. A. J. McIlhinney, unpublished work). Further work is needed to separate the milk oligosaccharides which inhibit binding of M3 and M24 to MFGM so that their epitopes can be more precisely defined.

From the observations that M8 can be prevented from binding to human MFGM by MFGM glycopeptides, but not by oligosaccharides, we have suggested that this antibody does not react with a simple sugar epitope. This conclusion has been

strengthened by studies on the binding of M8 to a high- M_r component of human MFGM called epithelial membrane antigen (EMA) (Ormerod *et al.*, 1983). These results also suggest that M8 binds to a complex epitope involving a short sequence of amino acids and sugars (M. G. Ormerod, J. H. Westwood & K. Steele, personal communication).

Many monoclonal antibodies raised against the human MFGM have not had the epitopes to which they bind fully characterized. However, HMFG-1 and HMFG-2 (formerly 1.10.F3 and 3.14-A3; Burchell *et al.*, 1983), like the antibodies described here, have been shown to recognize carbohydrate epitopes. Furthermore, it has been shown that both these antibodies and M8 react with EMA (Ormerod *et al.*, 1984a). It is clear from these studies that HMFG-1, HMFG-2 and M8 recognize different epitopes on this molecule. These three antibodies also react with a large mucin-like protein, PAS-0, which has been isolated from human MFGM (Shimizu & Yamauchi, 1982) and is antigenically and immunochemically very similar to EMA (Ormerod *et al.*, 1984b).

Immunochemical studies have also been performed using three monoclonal antibodies to human MFGM raised by Ceriani *et al.* (1983). One antibody, BLMRL-HMFG-Mc5, recognizes an

epitope carried by a high- M_r glycoprotein which has similar properties to EMA and PAS-0. The other two, BLMRL-HMFG-Mc3 and BLMRL-HMFG-Mc2, bind HMFG membrane proteins of 46kDa and 70kDa respectively. It is not known however if these antibodies react with carbohydrate or protein epitopes but, from their pattern of reaction to human MFGM proteins, they would appear to be distinct from those described here.

The least well-characterized series of monoclonal antibodies to the human MFGM are those described by Hilken *et al.* (1982). These authors classified their antibodies into seven main groups based on their immunohistochemical and immunochemical properties. It was suggested, on the basis of Pronase- and periodate-sensitivity experiments, that some of these antibodies bound to carbohydrate epitopes (Hilken *et al.*, 1983) but the biochemical data presented was limited and accurate comparisons between their study and the present report are difficult.

M3, M8, M18, M24, HMFG-1, HMFG-2 and the Mam series of monoclonal antibodies have all been shown to have a heterogeneous pattern of immunocytochemical staining on both normal and malignant human breast tissue (Foster *et al.*, 1982a,b; Arklie *et al.*, 1981; Hilken *et al.*, 1983; Edwards & Brooks, 1984). It has been suggested that the heterogeneity of staining exhibited by these antibodies may reflect the existence of subsets of epithelial cells at different stages of differentiation (Edwards & Brooks, 1984). This interpretation is supported by a number of reports describing developmentally regulated carbohydrate antigens in both human (Niemann *et al.*, 1978; Feizi *et al.*, 1979; Watanabe *et al.*, 1979) and mouse (Kapadia *et al.*, 1981) systems. However, modulation of the expression of carbohydrate antigens alone may not provide a reliable indicator of cell differentiation, since post-translational modifications of carbohydrate may occur in the absence of new gene expression. Such modifications may be due to changes in the availability of cations, alterations in pH, or other local effects which alter the activity of glycosyltransferases or hydrolases (Gooi *et al.*, 1983a,b; Feizi, 1981). The expression of carbohydrate antigens, especially those carried by glycolipids, may also be altered during the cell cycle (Hakamori, 1975) or by changes in the membrane lipid composition as shown for the Rhesus antigen (Shinitzky & Sarajou, 1979). Evidence for subtle modulations in the expression of glycolipid antigens also comes from the study by Willison *et al.* (1982) on the detection of lactosylceramide and Forssman antigen in murine embryos and on a murine embryonal carcinoma cell line, using conventional antisera and a monoclonal antibody to the Forssman antigen.

These considerations imply that it is important to identify those monoclonal antibodies which recognize carbohydrate antigens, particularly when they are to be used to monitor cell differentiation or to define different cell populations. Carbohydrate-binding antibodies are not uncommon, as shown by the number of monoclonal antibodies raised to both human and rodent tissues which have been shown to react with carbohydrate determinants (Gooi *et al.*, 1981; Eisenbarth *et al.*, 1979; Williams *et al.*, 1982; Canevari *et al.*, 1983; Willison & Stern, 1978; Cahan *et al.*, 1982; Pukel *et al.*, 1982).

We would suggest that the methods described here, namely the use of glycopeptides in competition assays, and the testing of glycolipids isolated from the immunizing material in direct binding assays, could be more generally applied to facilitate the identification of carbohydrate-binding antibodies. In those instances where the availability of immunizing material is limiting, we have found that preliminary screening of fusions by direct binding assays on glycolipids from human meconium and on red blood cells from donors with different blood groups has provided an alternative and effective way of identifying antibodies reacting with human carbohydrate antigens.

In conclusion we have shown that the monoclonal antibodies M3, M8, M18 and M24 all bind to determinants that involve carbohydrate and that M3 and M24 react with such determinants on both glycoproteins and glycolipids of the human MFGM. Comparisons made with the other monoclonal antibodies raised against MFGM suggest that these recognise different determinants from those which bind M3, M8, M18 and M24.

We thank Dr. P. Edwards and Mrs. I. Brooks for supplying the monoclonal antibodies, Professor R. U. Lemieux for providing the saccharides with X and Y antigenic structures, and Dr. J. Magnani for his advice on establishing the lipid binding assays. In particular we wish to thank Mrs. D. E. Mundy (Lactation Sister), Maternity Department, Kingston Hospital, Kingston-upon-Thames, Surrey, U.K., for providing us with human milk, Dr. D. Solter for providing SSEA-1 and Dr. K. Willison who supplied rabbit anti-Forssman serum. We are also indebted to Dr. A. Bukhari for the sugar analyses.

References

- Arklie, J., Taylor-Papadimitriou, J., Bodmer, W., Egan, M. & Millis, R. (1981) *Int. J. Cancer* **28**, 23-29
- Ashwell, G. (1966) *Methods Enzymol.* **8**, 93-96
- Bargmann, W. & Knoop, A. (1959) *Z. Zellforsch.* **49**, 344-388
- Burchell, J., Durbin, H. & Taylor-Papadimitriou, J. (1983) *J. Immunol.* **131**, 508-513
- Burnette, W. N. (1981) *Anal. Biochem.* **112**, 195-203

- Cahan, L. D., Irie, R. F., Singh, R., Cassidenti, A. & Paulson, J. C. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7629–7633
- Canevari, S., Fossati, G., Balsari, A., Sonnino, S. & Colnaghi, M. I. (1983) *Cancer Res.* **43**, 1301–1305
- Ceriani, R. L., Thompson, K., Peterson, J. A. & Abraham, S. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 582–586
- Ceriani, R. L., Peterson, J. A., Lee, J. Y., Moncada, R. & Blank, E. W. (1983) *Somatic Cell Genet.* **9**, 415–427
- Clamp, J. R., Bhatti, T. & Chambers, R. E. (1971) *Methods Biochem. Anal.* **19**, 229–250
- Edwards, P. A. W. (1983) *Biochem. Soc. Trans.* **11**, 171–172
- Edwards, P. A. W. & Brooks, I. M. (1984) *J. Histochem. Cytochem.* **32**, 531–537
- Eisenbarth, G. S., Walsh, F. S. & Nirenberg, M. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4913–4917
- Esselman, W. J., Laine, R. A. & Sweeley, C. C. (1972) *Methods Enzymol.* **28**, 140–156
- Feizi, T. (1981) *Trends Biochem. Sci.* **6**, 333–335
- Feizi, T., Childs, R. A., Watanabe, K. & Hakomori, S. (1979) *J. Exp. Med.* **149**, 975–980
- Finne, J. & Krusius, T. (1982) *Methods Enzymol.* **83**, 269–277
- Foster, C. C. (1983) *Biochem. Soc. Trans.* **11**, 299–300
- Foster, C. S., Edwards, P. A. W., Dinsdale, E. A. & Neville, A. M. (1982a) *Virchows Arch.* **394**, 279–293
- Foster, C. S., Dinsdale, E. A., Edwards, P. A. W. & Neville, A. M. (1982b) *Virchows Arch.* **394**, 295–302
- Franke, W. W., Heid, H. W., Grund, C., Winter, S., Freudenstein, C., Schmid, E., Jarasch, E.-D. & Kennan, T. W. (1981) *J. Cell Biol.* **89**, 485–494
- Freudenstein, C., Kennan, T. W., Eigel, W. N., Sasaki, M., Stadler, J. & Franke, W. W. (1979) *Exp. Cell Res.* **118**, 277–294
- Gooi, H. C., Feizi, T., Kapadia, A., Knowles, D. B., Salter, D. & Evans, M. J. (1981) *Nature (London)* **292**, 156–158
- Gooi, H. C., Uemara, K., Edwards, P. A. W., Foster, C. S., Pickering, N. & Feizi, T. (1983a) *Carbohydr. Res.* **120**, 293–302
- Gooi, H. C., Williams, L. K., Uemara, K., Hounsel, E. F., McIlhinney, R. A. J. & Feizi, T. (1983b) *Mol. Immunol.* **20**, 607–613
- Gore, M. E., Bunnage, H. J. & McIlhinney, R. A. J. (1984) *Eur. J. Immunol.* **14**, 344–349
- Hakomori, S.-I. (1975) *Biochim. Biophys. Acta* **417**, 55–89
- Heyderman, E., Steele, K. & Ormerod, M. G. (1979) *J. Clin. Pathol.* **32**, 35–39
- Hilkens, J., Buijs, F., Hilgers, J., Hageman, P. H., Sonnenberg, A., Koldovsky, U., Karande, K., Hoeven, R. P., Feltkamp, C. & Van de Rijn, J. M. (1982) *Protides Biol. Fluids* **29**, 813–817
- Hilkens, J., Hilgers, J., Buijs, F., Hageman, P. H., Schol, P., Van Doornewaard, G. & Van Den Tweel, J. (1983) *Protides Biol. Fluids* **31**, 1013–1016
- Imam, A., Laurence, D. J. R. & Neville, A. M. (1981) *Biochem. J.* **193**, 47–54
- Kapadia, A., Feizi, T. & Evans, M. J. (1981) *Exp. Cell Res.* **131**, 185–195
- Köhler, G. & Milstein, C. (1975) *Nature (London)* **256**, 495–497
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Magnani, J. L., Nilsson, B., Brockhaus, M., Zopf, D., Steplewski, Z., Koprowski, H. & Ginsburg, V. (1982) *J. Biol. Chem.* **257**, 14365–14369
- Niemann, H., Watanabe, K., Hakomori, S., Childs, R. A. & Feizi, T. (1978) *Biochem. Biophys. Res. Commun.* **81**, 1286–1293
- Ormerod, M. G., Steele, K., Westwood, J. H. & Mazzini, M. N. (1983) *Br. J. Cancer* **48**, 533–541
- Ormerod, M. G., Steele, K., Edwards, P. A. W. & Taylor-Papadimitriou, J. (1984a) *J. Exp. Pathol.*, in the press
- Ormerod, M. G., McIlhinney, R. A. J., Steele, K. & Shimizu, M. (1984b) *Mol. Immunol.*, in the press
- Pukel, C. S., Lloyd, K. O., Travassos, L. R., Dippold, W. G., Oetgen, H. F. & Old, L. J. (1982) *J. Exp. Med.* **155**, 1133–1147
- Sacke, R. G., & Heald, C. W. (1974) in *Lactation* (Larson, B. L. & Smith, V. R., eds.), vol. 2, pp. 147–189, Academic Press, New York
- Shimizu, M. & Yamauchi, K. (1982) *J. Biochem. (Tokyo)* **91**, 515–524
- Shinitzky, M. & Sarajou, M. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4438–4440
- Sloane, J. P. & Ormerod, M. G. (1981) *Cancer* **47**, 1786–1795
- Smith, D. F., Zopf, D. A. & Ginsberg, V. (1978) *Anal. Biochem.* **85**, 602–608
- Solter, D. & Knowles, B. B. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5565–5569
- Svennerholm, L. (1963) *J. Neurochem.* **10**, 613
- Taylor-Papadimitriou, J., Peterson, J. A., Arklie, J., Burchell, J., Ceriani, R. L. & Bodmer, W. F. (1981) *Int. J. Cancer* **28**, 17–21
- Watanabe, K., Hakomori, S., Childs, R. A. & Feizi, T. (1979) *J. Biol. Chem.* **254**, 3221–3228
- Williams, L. K., Sullivan, A., McIlhinney, R. A. J. & Neville, A. M. (1982) *Int. J. Cancer* **30**, 731–738
- Willison, K. R. & Stern, P. L. (1978) *Cell* **14**, 785–792
- Willison, K. R., Karol, R. A., Suzuki, A., Kundu, S. K. & Marcus, D. M. (1982) *J. Immunol.* **129**, 603–609
- Yu, R. K. & Ledeen, R. W. (1972) *J. Lipid Res.* **13**, 680–686