The EMBO Journal vol.11 no.11 pp.3845-3855, 1992

Involvement of a neutral glycolipid in differential cell adhesion in the *Xenopus* blastula

A.P.Turner[1], D.Brown[2,4], J.Heasman[1], G.M.W.Cook[3], J.Evans[1], L.Vickers[1] and C.C.Wylie[1,5]

[1]The Wellcome Trust/CRC Institute of Cancer and Developmental Biology, Tennis Court Road, Cambridge CB2 1QR, [2]St George's Hospital Medical School, Cranmer Terrace, Tooting, London SW17 0RE and [3]Department of Anatomy, Cambridge University, Downing Street, Cambridge CB2 3DY, UK

[4]Present address: Department of Immunomodulation, Celltech Ltd, 216 Bath Road, Slough SL1 4EN, UK

[5]Corresponding author

Communicated by R.A.Dwek

Many different molecular species mediate cell adhesion during embryonic development. These can have either protein or carbohydrate functional groups, which can act in either a homophilic or a heterophilic manner, and often in concert. We report here that a monoclonal antibody, M4B, raised against *Xenopus* blastomere membranes, inhibits the calcium-dependent adhesion of dissociated blastomeres. M4B maintains its inhibitory effect on adhesion when converted into univalent fragments, and specifically affects calcium-dependent adhesion. The antigen is regulated in both space and time during early development. It is found on cell surfaces throughout the egg to blastula stages, but is more concentrated on cells in the animal and marginal zones of the blastula. It is dramatically downregulated during gastrulation, and becomes largely restricted to gut epithelium by the larval stages. We show also that M4B function is spatially differentiated at the blastula stage, since it inhibits the aggregation of dissociated animal cells to a greater extent than vegetal cells. This membrane antigen may therefore play a role in the differential adhesion observed between different regions of the blastula, and which we presume to underlie the segregation of the primary germ layers during gastrulation. M4B recognizes a complex of plasma membrane glycolipids. Periodate treatment destroys the ability of these glycolipids to react with the antibody, indicating that the epitope resides in the carbohydrate moiety of the glycolipids. Chemical characterization shows that it is a neutral glycolipid, and that the major component is of the glycoglycerolipid, rather than the more common glycosphingolipid class. Blocking experiments with oligosaccharides of defined structure, and antibody crossreactivity show that the M4B antibody does not recognize several known embryonic carbohydrate antigens. These results demonstrate that M4B antibody recognizes a novel group of developmentally regulated glycolipids which function in calcium-dependent cell-cell adhesion in the *Xenopus* blastula.

Key words: cell adhesion/cell surface carbohydrate/glycolipid/*Xenopus*

Introduction

Gastrulation in vertebrate embryos represents the most profound change in morphology. In *Xenopus*, the blastula is a hollow ball of cells, with no obvious tissue boundaries. Six hours later, the gastrula contains three clearly segregated primary germ layers. The cell surface changes that underly this tissue segregation start at the blastula stage, since cell transplantation (Heasman *et al.*, 1984) and cell sorting (Turner *et al.*, 1989) experiments show that differential adhesion appears during the blastula stage in *Xenopus*.

Cell surface glycoconjugates have long been known to play a role in cell adhesion and recognition. Early studies with lectins (reviewed by Sharon and Lis, 1989), glycosylation inhibitors (Oppenheimer *et al.*, 1969) and mutants (Francis *et al.*, 1985), sugar degrading reagents such as periodate (Moscona, 1962) and neuraminidase (Lloyd, 1975), all point to a general role of carbohydrate groups in these cellular properties.

More recently, defined sugar groups have been shown to play tissue-specific roles in cell adhesion. The sulphated glucuronic acid antigen HNK-1 is thought to have an adhesive role in the developing nervous system (Künemund *et al.*, 1988). Early mouse embryos express a specific carbohydrate antigen known either as SSEA-1 (stage-specific embryonic antigen-1; Solter and Knowles, 1978), or Le^X (Lewis-X, Eggens *et al.*, 1989). Expression starts at the time of compaction in the early cleaving embryo. The anti-SSEA-1 antibody that first defined this antigen was found to bind to the sequence $Gal_{\beta 1\text{-}4}[Fuc_{\alpha 1\text{-}3}]GlcNAc$- (Gooi *et al.*, 1981). Oligosaccharides containing this sequence were subsequently found to block compaction (Bird and Kimber, 1984). One of these oligosaccharides, LNFP III (lacto-*N*-fucopentaose III) also inhibited the aggregation of F9 cells (Eggens *et al.*, 1989). A variety of evidence suggests that the Le^X antigen on F9 cells acts as a homophilic binding molecule, via Le^X-Le^X interactions (Eggens *et al.*, 1989). SSEA-1/Le^X is carried on a glycolipid in the early mouse embryo and F9 cells. In the latter it is also carried on a large glycoprotein known as embryoglycan (Childs *et al.*, 1983; Eggens *et al.*, 1989). Its adhesive role requires calcium ions.

Recently, a novel class of cell-cell adhesion molecules has been identified, known as selectins. They are a group of structurally related transmembrane proteins expressed on leukocytes, the endothelial linings of blood vessels and elsewhere, that mediate calcium-dependent adhesion via a lectin-carbohydrate interaction (reviewed by Stoolman, 1989; Osborn, 1990). Selectins share, amongst other sequences, a single domain homologous to that of calcium-dependent animal lectins (Drickamer, 1988). Several specific carbohydrate sequences are now known to act as the ligands of selectins (see Brandley *et al.*, 1990; Feizi, 1991 for reviews). In particular, the SSEA-1/Le^X antigen has been found to bind to P-selectin (Larsen *et al.*, 1990), and the sialylated version of this sequence may be the ligand for E-selectin (Phillips *et al.*, 1990; Walz *et al.*, 1990).

In this paper, we demonstrate the presence of a group of cell surface glycolipids in early *Xenopus* embryos recognized by monoclonal antibody M4B. The expression of these is regulated both spatially and temporally, and we present evidence that they are involved in calcium-dependent adhesion between blastomeres. Furthermore, the antigen is differentially expressed in the blastula, in a distribution that is mimicked by the differential effect of the antibody on blastomere adhesion.

Results

Monoclonal antibodies raised against Xenopus blastula membranes reveal surface molecules involved in cell adhesion

Monoclonal antibodies were raised against both whole blastomeres or blastomere membrane preparations, from late blastulae (stage 9 of Nieuwkoop and Faber, 1967). Many of these antibodies cross-reacted with blastomere membranes (Figure 1), and these were then screened for the capacity to block the calcium-dependent reaggregation of blastomeres after disaggregation in calcium- and magnesium-free saline. A simple scoring system was devised to test the degree to which an antibody blocked aggregation. Scores of 0−3, in which 0 = no aggregation and 3 = aggregation to a smooth sphere, were given by two independent observers according to a set of standard criteria (shown in Figure 2). Sample results after 1 h aggregation assays are shown for two antibodies: M4B, which blocks aggregation, and 9V70 which does not. M4B blocked aggregation at concentrations of whole Ig down to 10 μg/ml.

To confirm that it is calcium-dependent adhesion which is affected by M4B, blastomeres were treated with trypsin in the presence of calcium, to remove any calcium-independent but retain calcium-dependent adhesion mechanisms (Takeichi *et al.*, 1979; Nomura *et al.*, 1986). Subsequent to this treatment, a 1:1 dilution of M4B supernatant reduced reaggregation of stage 9 blastomeres over 2 h to 1.4 (n = 4, SD = 0.63) as against 2.6 (n = 4, SD = 0.48) for controls exposed to 9V70 supernatant.

All the monoclonal antibodies generated in this study were of IgM class, which are large and multivalent, so could potentially mask or cause the redistribution of other surface components. We therefore fractionated the M4B antibody using the Matthew and Reichardt method (1982). This procedure yields a variety of fragments, the most abundant of which is an active monovalent fragment of mol. wt 110 kDa, composed of one intact heavy and one light chain. Whole M4B IgM was digested with chymotrypsin-free trypsin, and fragments from this digestion were fractionated by HPLC, using size selection by gel filtration (Figure 3). The most abundant fragment (peak 'a' in Figure 3A) was found in non-reducing polyacrylamide gels to be the correct size, i.e. 110 kDa, for a monovalent fragment (Matthew and Reichardt, 1982; Figure 3B). This fragment blocked aggregation of blastomeres as efficiently as whole IgM. Figure 3C shows the degree of inhibition after 2.5 h (averaging score 1.5 on the above scale) using a concentration of 50 μg/ml. It is therefore unlikely that the results using the whole antibody could have come about by non-specific interference with other surface molecules.

Expression of the M4B surface antigen is developmentally regulated

M4B was used to stain tissue sections of ovary and different developmental stages up to the swimming larva, by which

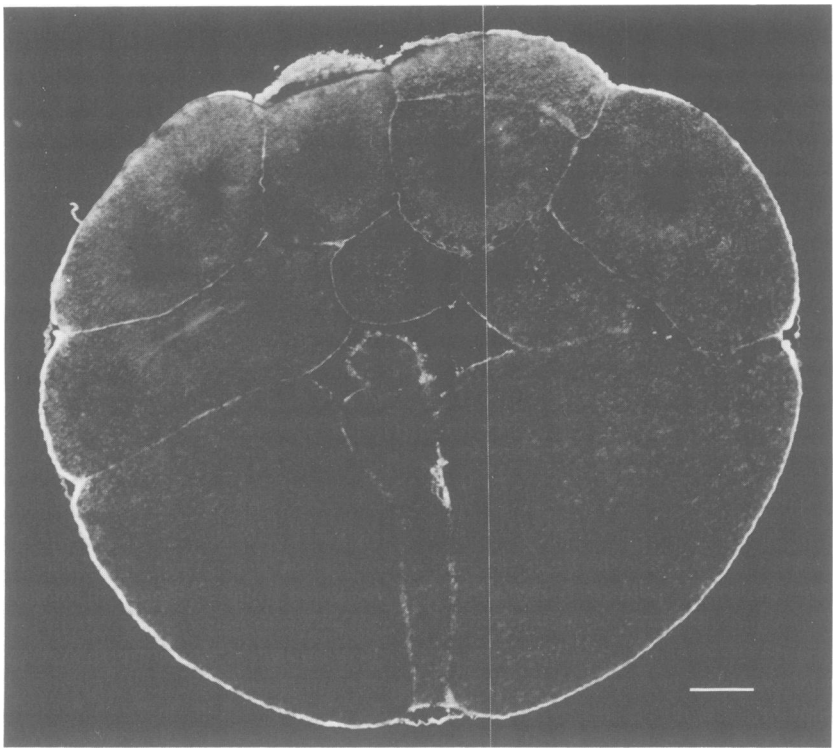
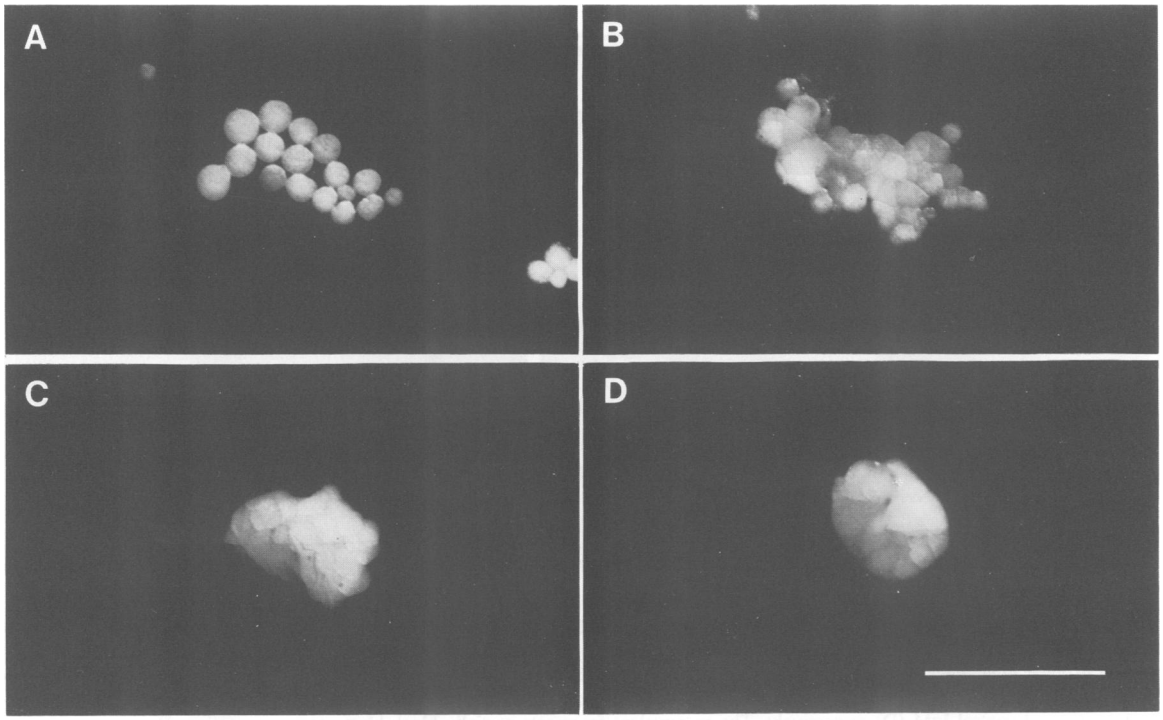


Fig. 1. Monoclonal antibody M4B stains blastomere membranes. A section through an early blastula (stage 6 according to Nieuwkoop and Faber, 1967), stained with M4B supernatant. The outer surface of the embryo (including the vitelline membrane) and all blastomere surfaces are stained. Bar = 100 μm.

time most differentiated tissues are present. Staining was seen in both early, previtellogenic oocytes (Figure 4A), as well as full-grown oocytes (Figure 4B). Full-grown oocytes showed cytoplasmic as well as surface staining. After fertilization, M4B stained all blastomere cell surfaces during the egg to blastula stages (Figures 1 and 4C). After gastrulation, cell surface staining declined rapidly, until by the tailbud stage it was confined to cells lining the lumen of the early gut (Figure 4D). In other areas of the tailbud stage embryo, little or no cell surface staining was seen, although a population of positively staining cytoplasmic vesicles was present in some tissues (Figure 4E). At the early swimming tadpole stage, surface staining in the epithelium lining the gut had become very intense (Figure 4F). Most other tissues were negative, with the exception of rare single positive cells. At this stage, some of the primordial germ cells migrating from the gut to the genital ridges expressed the M4B antigen (Figure 4G). Control sections at all stages, in which the primary antibody was omitted, or replaced by a non-specific mouse IgM, were negative (Figure 4H).



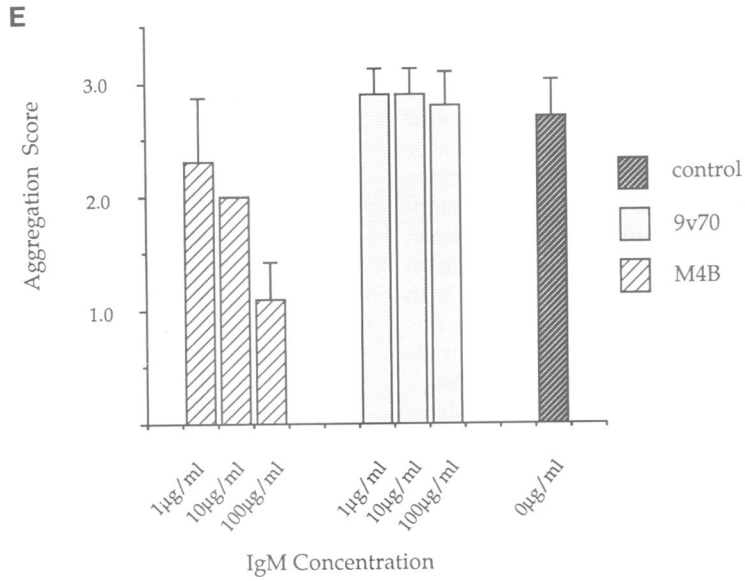


Fig. 2. Inhibition of reaggregation by monoclonal antibody M4B. (**A**–**D**) Show sample aggregates with aggregation scores of 0–3 inclusive. This scoring system was used to assay the efficiency of different monoclonal antibodies in blocking calcium-dependent reaggregation of blastomeres. Bar = 1 mm. (**E**) Shows results of such an assay. Between six and 10 duplicate aggregates were scored after 1 h in medium alone, or containing M4B or 9V70 monoclonal antibodies. M4B inhibits blastomere aggregation whereas 9V70 does not. Both antibodies stain blastomere surfaces at the same antibody concentrations. Error bars are shown for each score. All aggregates in 10 μg/ml M4B scored the same, so the error is zero.

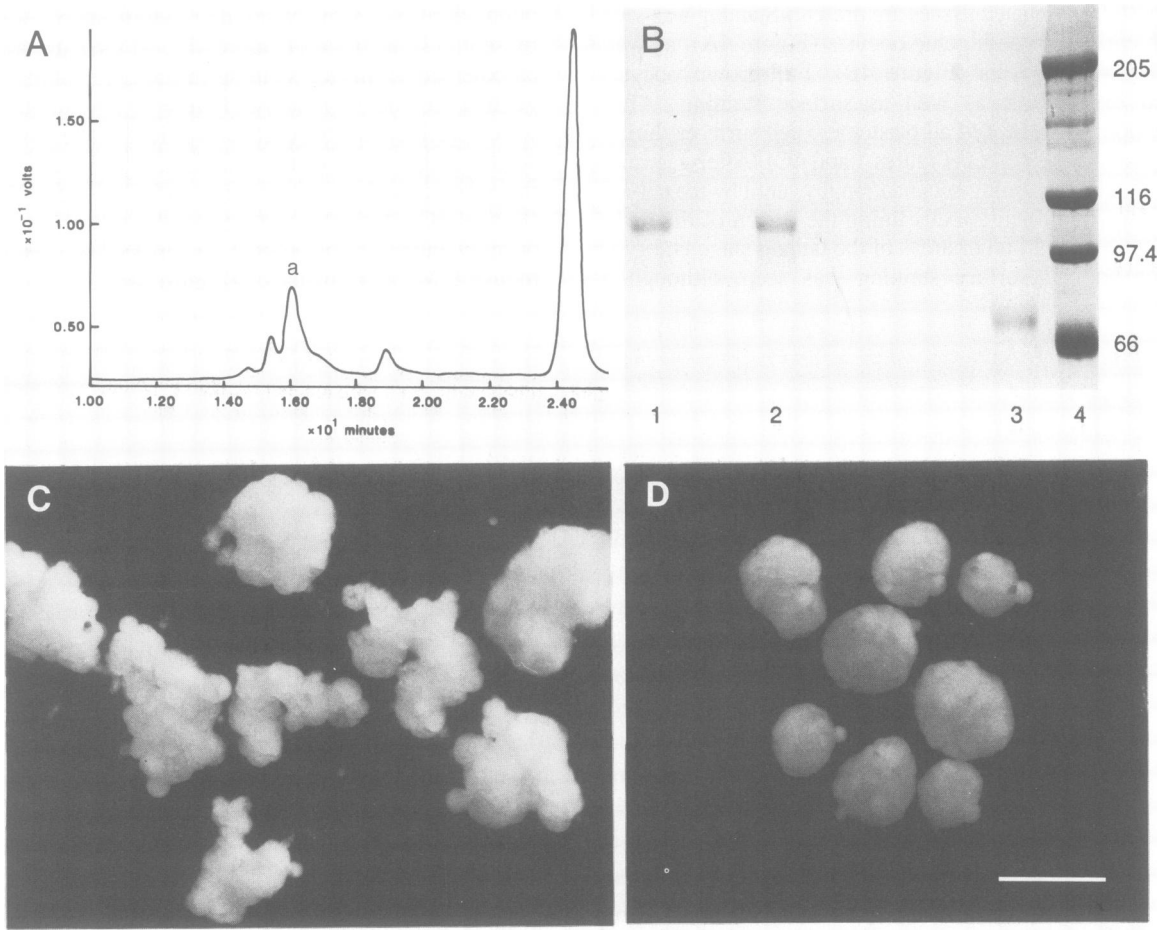


Fig. 3. Inhibition of blastomere aggregation by monovalent fragments of M4B. (**A**) Separation by HPLC gel filtration of a digest of whole M4B IgM. Peak 'a' is indicated. (**B**) Shows non-reducing SDS–polyacrylamide gel analysis of peak 'a' (track 1) and the whole digest (track 2), stained with Coomassie brilliant blue. Peak 'a' has an apparent M_r of 110. Track 3 shows reduced peak 'a' protein. The single band seen is the correct M_r (~70) for IgM heavy chain. Track 4 = mol. wt markers; (**C**) and (**D**) show the degree to which blastomeres reaggregate in 2.5 h in 50 μg/ml of peak 'a' protein (C) and control IgM (D) respectively. The monovalent fragments partially block blastomere aggregation. Bar = 1 mm.

M4B antigen is a carbohydrate, carried on a neutral glycolipid, in the Xenopus blastula

Western blots and immunprecipitations with several of the aggregation-blocking monoclonals, including M4B, failed to reveal any crossreaction with protein from oocytes or embryos. We therefore tested for crossreactions with surface glycolipids, using high performance thin layer chromatography (HPTLC) on silica gel-covered aluminium sheets.

Figure 5A shows a blastula crude lipid preparation separated on a glass-backed silica gel plate stained with orcinol to show all the glycolipid species resolved by this technique. Figure 5B shows an aluminium-backed chromatogram of the same preparation stained with the M4B antibody, which reacted with a complex of slow-moving bands whose position relative to the other glycolipids is indicated on the orcinol-stained plate. The two plates cannot be directly aligned, since they were developed for different times. When HPTLC plates were pre-treated with 20 mM sodium periodate (modified from Woodward *et al.*, 1985) before staining with M4B, the crossreacting bands were eliminated (Figure 5C). This indicates that M4B reacts with the carbohydrate moiety of the glycolipids. This conclusion is reinforced by the elimination of M4B immunofluorescent staining of blastula sections by periodate treatment (data not shown). Several of the monoclonal antibodies isolated separately during this study (S91, M3 and M4B) reacted with the same complex of glycolipids. They also had identical effects on blastomere aggregation and staining patterns on histological sections. For convenience they will all be referred to as M4B, since it was this particular antibody that is used almost entirely in these experiments, and it was the first antibody found.

Figure 5D shows the crossreaction of M4B with glycolipid extracts from different regions and stages of embryos. The same group of bands was seen throughout the blastula and gastrula stages, disappearing almost completely by the tailbud stage, and reappearing in the gut at the larval stage. This result correlates precisely with the immunofluorescent staining patterns described above (Figure 4). Figure 5D also shows M4B-stained plates of lipid extracts from dissected animal and vegetal blastula regions. The dissected pieces excluded the marginal zone of the embryo. The same major bands were seen in both. Control antibodies used in these staining reactions were a monoclonal anti-actin, a non-specific IgM and monoclonal antibodies raised in this study that stain blastomere surfaces but do not recognize glycolipids. All were completely negative (e.g. Figure 5B, track 2).

Crude lipid extracts from blastulae were subjected to Folch partition, and the species reacting with M4B were found in

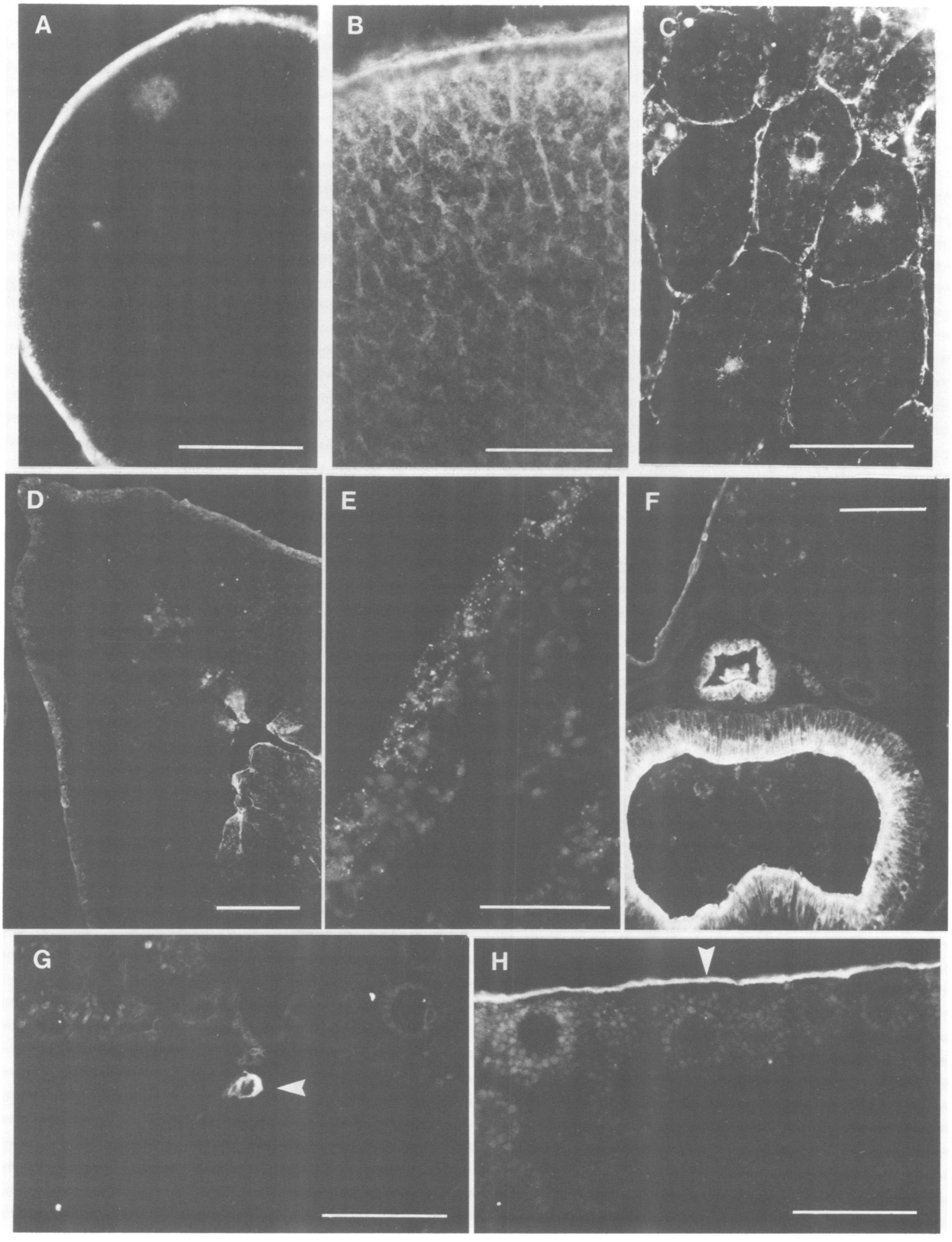


Fig. 4. Developmental regulation of M4B expression. (**A**) Section through previtellogenic oocyte, showing strong cortical and surface staining. (**B**) Section through full-grown oocyte. Strands of cytoplasmic staining are evident, as well as a prominent signal at the oocyte surface. (**C**) Section through stage 9 (late blastula) embryo. Cell membranes continue to stain strongly, while some cytoplasmic staining also remains. (**D**) Transverse section through stage 26 (tailbud) embryo. Membrane staining is restricted to endodermal cells lining the early gut. Some staining of cytoplasmic vesicles in the epidermis is also evident. (**E**) Transverse section through stage 26 dorsal region at higher magnification to show the vesicular nature of staining in epidermal cells. (**F**) Cross section through stage 45 tadpole gut coils. Membranes of gut epithelial cells stain prominently, especially on the lumenal surface. (**G**) Stage 45 tadpole sectioned through the mid-gut, showing positive staining of a migratory primordial germ cell (arrowhead). (**H**) Control stage 9 blastula (no primary antibody). No cell surface signal is present, but the vitelline membrane stains non-specifically (arrowhead). Bar = 100 μm.

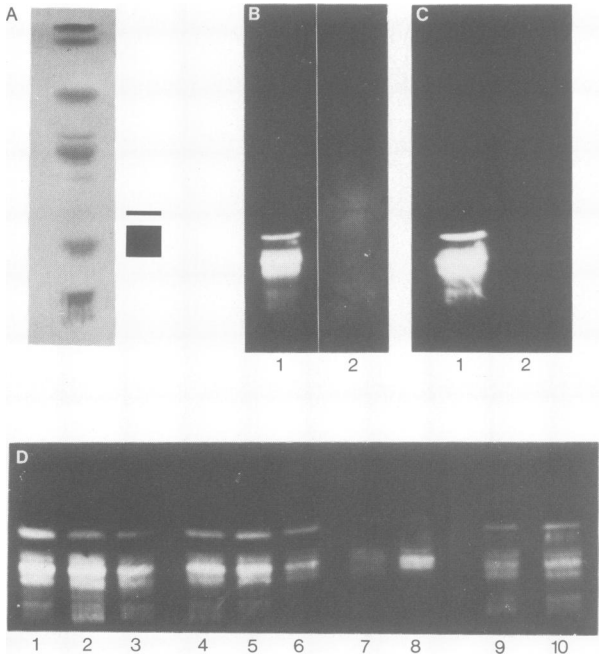


Fig. 5. The M4B antigen is the carbohydrate moiety of a membrane glycolipid. (**A**) TLC separation of crude blastula glycolipid extract (four embryos worth) stained with orcinol reagent to show the positions of the major glycolipid species. (**B**) HPTLC plate (four embryos worth per lane) stained with M4B (track 1) or 9V70 (track 2). M4B crossreacts with a small group of slow moving glycolipids. The relative positions of these are marked in A, as calculated from the relative mobilities of glycolipid standards. (**C**) Shows the effect of treating the TLC plates with 20 mM periodate before staining with M4B. Two duplicate samples are shown, one without (track 1) and one with (track 2) pretreatment with periodate. (**D**) HPTLC plates of membrane glycolipid preparations from different stages stained with M4B. The tracks are as follows: 1–3, early, mid and late blastula; 4 and 5, early and late gastrula; 6, neurula, 7, tailbud; 8, gut of swimming larva; 9 and 10, animal and vegetal pieces from late blastulae. Lane loadings were adjusted to the equivalent of approximately four stage 9 embryos worth per lane.

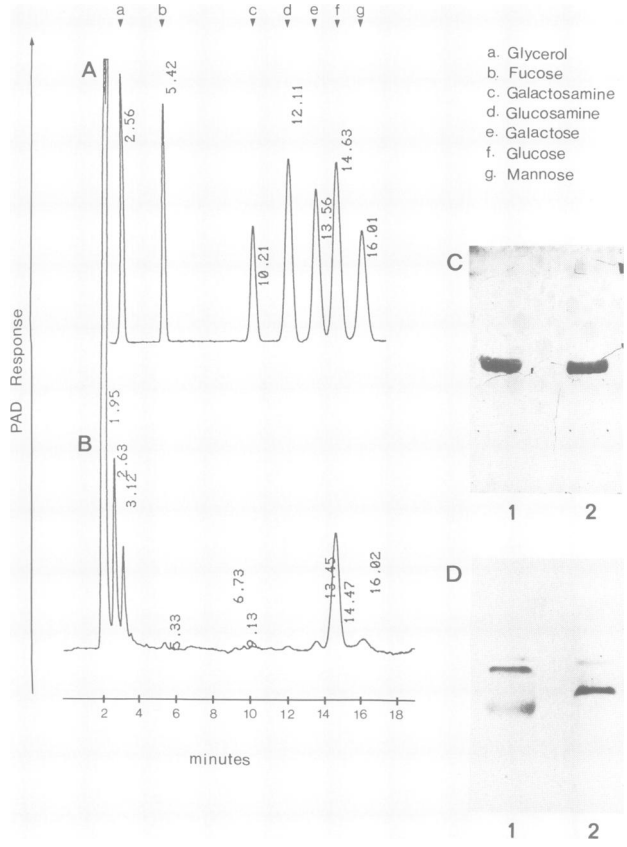


Fig. 6. Chemical nature of the M4B antigen complex. (**A**) Shows two HPLC separations on a CarboPac PA1 column, one of glycerol (2.3 nmol) and the other of a mixture of standard monosaccharides; fucose (1.5 nmol), galactosamine (1.2 nmol), glucosamine (2.1 nmol), galactose (2.1 nmol), glucose (2.2 nmol) and mannose (1.8 nmol) in 15 mM NaOH. Post-column addition of 0.3 M NaOH was performed to optimize base line stability and PAD detector (gold electrode) sensitivity. (**B**) Fractionation on CarboPac PA1 column of acid hydrolysate of M4B antigen purified from TLC plates (see Materials and methods). The principal peaks are those of glycerol (1.7 nmol, R_t 2.63 min) and glucose (1.4 nmol, R_t 14.47 min), with traces of other monosaccharides. This sample was run immediately after the standards and under exactly the same conditions. In all cases, the PAD cell was set at an output range of 1 KnA. Retention time (R_t, in min) is given for each peak. (**C**) Orcinol-stained HPTLC separation, using the solvent mixture chloroform:methanol:0.02%$CaCl_2 \cdot 2H_2O$ (60:40:9 v/v/v) of a standard asialoGM1 (100 μg, track 1) and the same preparation previously subjected to alkali-catalysed methanolysis (track 2). The sample is unaltered by this treatment. (**D**) In contrast, a preparation of M4B antigen, treated identically, has its mobility shifted by alkali-catalysed methanolysis (track 2) compared with the untreated preparation (track 1).

the lower organic phase. This phase was fractionated by silicic acid chromatography. M4B-reactive species were found in the fraction in which phospholipids are known to be recovered. TLC analysis showed that this fraction contained phospholipid (Rf 0.69, 0.55, 0.25 and 0.16) as assessed by the Dittmer–Lester molybdate reagent. However, when the carbohydrate moiety of the putative M4B glycolipid was acetylated with pyridine acetic anhydride and the 'phospholipid' fraction (i.e. the methanol fraction from silicic acid chromatography) fractionated on Florisil, it was possible to recover the glycolipid free of phospholipid by elution with 1,2-dichloroethane:acetone (1:1 v/v). The glycolipid was deacetylated by a brief (10 min) treatment with 0.5 M sodium methoxide at 50°C and analysed by HPTLC. Material reacting with M4B was readily detected in the complete absence of phospholipid as assessed by the Dittmer–Lester reagent. No species reacting with M4B was found in the upper aqueous phase, and DEAE chromatography failed to concentrate any antibody-reactive species. DEAE column chromatography separates acidic compounds, sulphatides and gangliosides, from less acidic or nonpolar compounds. We conclude from this that the M4B antigen is a neutral glycolipid.

Glycolipids previously purified on preparative TLC plates were acid hydrolysed (1 M HCl at 100°C for 12 h) and the water-soluble products separated by HPLC using a CarboPac PA1 column eluted with 15 mM NaOH, with post-column addition of 0.3 M NaOH coupled to PAD detection (Figure 6A and B). Monosaccharides found were primarily glucose [retention time (R_t) 14.47 min] together with traces of components in the correct positions for fucose (R_t 5.33 min), galactose (R_t 13.45 min) and mannose (R_t 16.02 min). A standard glycosphingolipid (asialoGM1) when analysed by the same procedure, showed the expected distribution of galactosamine, galactose and glucose in the ratio of 1.3:2:1 (not shown).

Although sequencing of the carbohydrate moiety has not

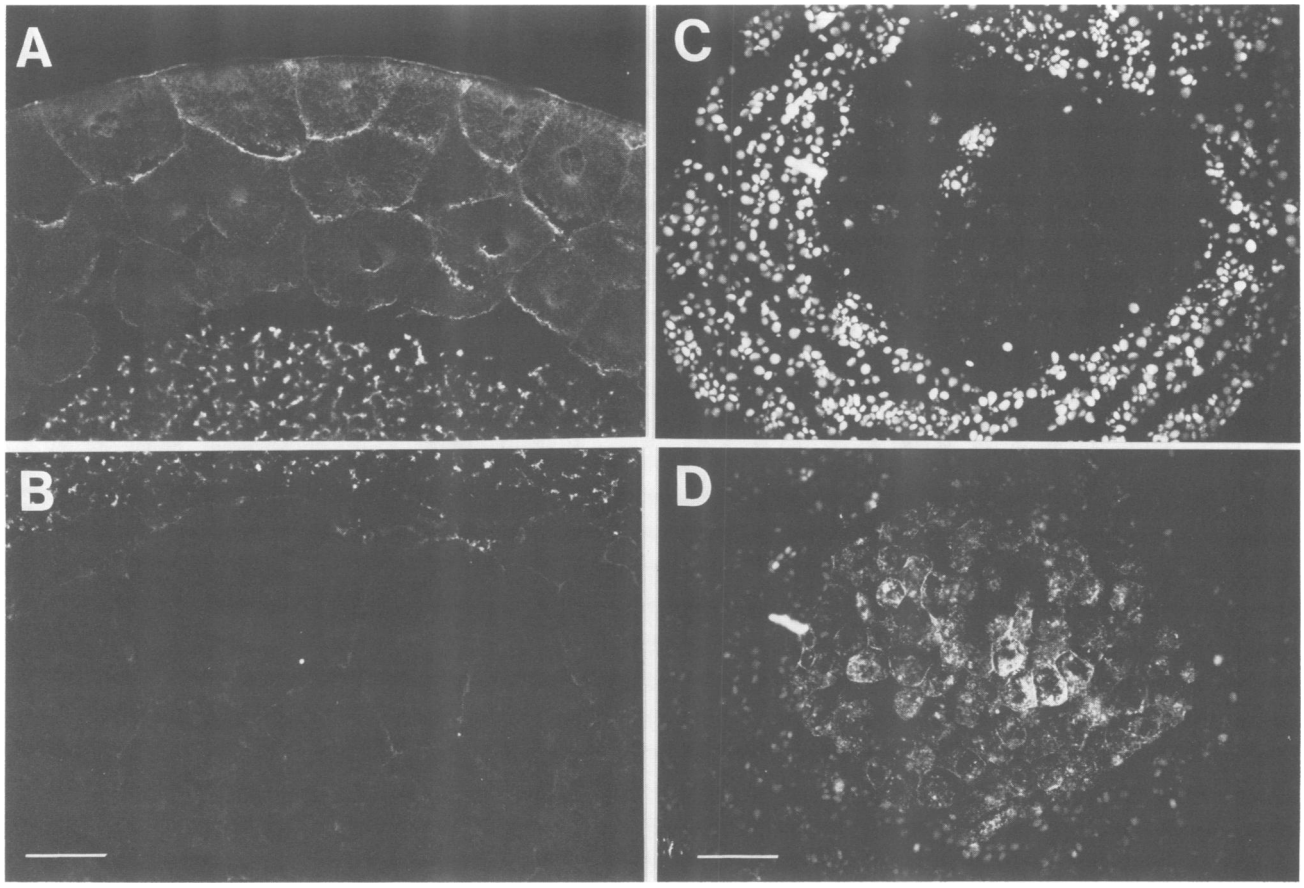


Fig. 7. Differential distribution of M4B antigen in the blastula. Fields of view from the animal (**A**) and vegetal (**B**) regions of a midblastula section stained with M4B. The exposure times, and subsequent printing times were identical in both cases. Both fields of view contain part of the blastocoel, which also contained M4B reactive material. Animal cells stain more brightly with the M4B antibody. This effect can be seen better in aggregates of animal cells mixed with vegetal cells and allowed to sort out. When animal cells are artificially directly juxtaposed with vegetal cells like this, they can be seen to contain much more M4B-reactive material. The same histological section is shown, photographed through the rhodamine filter set (**C**), to reveal the rhodamine-stained vegetal cells, and through the fluorescein filter set (**D**) to reveal the staining with M4B antibody. Bar = 50 μm (A and B), 100 μm (C and D).

yet been carried out, we wished to eliminate the possibility of crossreaction of the M4B antibody with the carbohydrate antigens known to be expressed in embryos of other species. M4B does not crossreact with either mouse embryonic stem cells or primordial germ cells, which are known to express the SSEA-1 antigen; whilst anti-SSEA-1, anti-HNK-1 and anti-SSEA-3 do not crossreact with glycolipids from *Xenopus* blastulae separated by TLC (data not shown). In addition, HPTLC analysis showed that M4B did not crossreact with sulphated glucuronic acid containing glycolipid isolated from human cauda equina, which did crossreact with the HNK-1 antibody. As a further test for SSEA-1, three structurally dissimilar oligosaccharides were tested for their ability to block M4B staining of *Xenopus* blastulae. These were: LNFP III = $Gal_{\beta 1\text{-}4}[Fuc_{\alpha 1\text{-}3}]GlcNAc_{\beta 1\text{-}3}Gal_{\beta 1\text{-}4}Glc$; LND1 = $Fuc_{\alpha 1\text{-}2}Gal_{\beta 1\text{-}3}[Fuc_{\alpha 1\text{-}4}]GlcNAc_{\beta 1\text{-}3}Gal_{\beta 1\text{-}4}Glc$; and LNT = $Gal_{\beta 1\text{-}3}GlcNAc_{\beta 1\text{-}3}Gal_{\beta 1\text{-}4}Glc$. LNFP III contains the SSEA-1 antigen, whilst the others are structurally distinct oligosaccharides recognized by other monoclonal antibodies. None of these oligosaccharides at concentrations of 1 mg/ml inhibited M4B staining of *Xenopus* blastulae. As a positive control, these three standard oligosaccharides were tested for their ability to inhibit the staining of mouse primordial germ cells in sections of 12.5 days post-coitum embryos. As expected, LNFP III inhibited the staining of PGCs by anti-SSEA-1, whereas the other oligosaccharides had no effect (data not shown).

Whilst fractionating acid hydrolysates of TLC-purified glycolipid, we observed an early eluting peak in the correct position for glycerol (Figure 6A and B). The presence of small quantities of glycerol in these hydrolysates was confirmed independently by a UV method using a glycerokinase-coupled reaction. Total glycerol found by HPLC was 0.6 μg, and in the same amount of material using the enzyme-based reaction was 0.8 μg. These data suggest that M4B antigen is a glycoglycerolipid, rather than the more common glycosphingolipid. The most useful diagnostic test for glycoglycerolipids (Murray and Narasimhan, 1990) in a lipid fraction is to carry out alkali-catalysed methanolysis using mild conditions (Levine *et al.*, 1975). Glycosphingolipids, which possess an amide bond linking the fatty acid to the long-chain base component of ceramide are not affected by mild alkali; however, the acyl bonds in glycoglycerolipids are readily cleaved. HPTLC chromatography of a standard glycosphingolipid, asialo-GM1 showed that its mobility was not altered by this treatment (Figure 6C). However, alkali-catalysed methanolysis of a blastula lipid fraction revealed that this procedure retarded the mobility of the major band of the M4B antigen complex compared with control, untreated lipid (Figure 6D). This suggests that the major component

of the M4B antigen complex is a glycoacylalkyl glyceride, since a glycodiacyl glyceride would have generated glycosyl glycerol, which, being quite polar, would barely have left the origin of the chromatogram. One curious observation was that the fastest moving, and least reactive band of the M4B complex was not retarded by alkalic-catalysed methanolysis, suggesting either that a small amount of the carbohydrate antigen is carried on a glycosphingolipid, or there is a weak crossreaction of the M4B antibdy with another carbohydrate.

M4B antigen is involved in differential cell adhesion at the blastula stage

Immunochemistry on sections through the middle of the blastula consistently revealed that staining is more intense in the animal than the vegetal hemisphere (Figure 7A and B). This is particularly well seen when isolated animal cells are mixed with isolated vegetal cells, and allowed to aggregate by the addition of calcium. After 2–3 h, the cells have aggregated into a spherical ball, and have sorted out, with the animal cells in the middle of the aggregate. Staining of these aggregates with M4B (Figure 7C and D) shows that the central animal cells stain much more strongly than the outer vegetal cells. In order to test whether M4B antigen is functionally different in the two regions, we tested the effect of the same concentration of M4B on animal and vegetal cells separately. In five separate experiments, we compared the time course of aggregation of animal and vegetal cells from the mid-late blastula stage, in the presence and absence of M4B. The results of this are shown in Table I. There is no difference between the aggregation of animal and vegetal cells after 3 h in the absence of antibody. However, there is a statistically significant difference in its presence; animal blastomere aggregation is inhibited to a significant degree, whereas vegetal cells are not.

Discussion

In this study we show that a monoclonal antibody, M4B, made against blastomere membranes, blocks blastomere adhesion and recognizes a carbohydrate antigen carried on a discrete group of neutral glycolipids. The M4B antibody does not cross-react with proteins as shown by immunoprecipitation or by Western blotting. This does not exclude the possibility that the antigen could also be carried on a protein, however, we have been unable to demonstrate one. The close correlation between the pattern of immunostaining and the presence of crossreactive glycolipids as seen by HPLTC suggest that these are the main carriers of the M4B antigen. Other vertebrate embryos also express carbohydrate antigens carried on glycolipids which are expressed in developmentally regulated patterns. Examples include the stage-specific embryonic antigen series in mouse embryos (see Fenderson *et al.*, 1990 for review), Forssman antigen of human and mouse (Willison *et al.*, 1982), the mouse I and i antigens (Kapadia *et al.*, 1981) and the HNK-1/L2 antigen of mammalian nervous systems (Schwarting *et al.*, 1987; see Jessell *et al.*, 1990 for review). The expression pattern of M4B antigen most closely resembles mammalian SSEA-1. Both are expressed at the blastula stage, and then re-expressed at later stages on different issues. However, we show here by a combination of oligosaccharide blocking and antibody crossreactivity studies that the two antigens are different carbohydrate groups.

The M4B antigen is differentially expressed, both spatially and temporally during early development. It disappears from most tissues of the embryo during gastrulation, as shown both by immunocytochemistry and HPTLC chromatography. This could be due either to its elimination from the surface, or by masking of the antigenic site. The latter would have to be irreversible, since the crossreactivity does not reappear in the majority of tissues of the later embryo. The disappearance of M4B antigen may exemplify the replacement of early embryo adhesion systems by more regionally specific combinations of adhesion molecules, such a neural cell adhesion molecule (N-CAM; Kintner and Melton, 1987; Levi *et al.*, 1987). M4B stains the outer surfaces of both eggs and blastulae. This is a perplexing observation, since this surface of the embryo is non-adhesive for other embryos or isolated blastomeres (Roberson and Armstrong, 1980), and other adhesion molecules present at the blastula stage are not found on the outer surface (Angres *et al.*, 1991). It may be that M4B antigen function can be modulated at different sites of its expression.

As yet we do not have detailed structural information on the sequence of saccharides in the M4B antigen. Chemical analysis shows, however, that it is carried on a complex of neutral glycolipids which migrate close together on HPTLC plates. Alkali-catalysed methanolysis, and the direct demonstration of glycerol, suggest that the principal

Table I. Inhibition of blastomere aggregation. Comparison of animal versus vegetal blastomere aggregation after 3 h in the presence of M4B or control antibody

Experiment	A	B	C	D
1	2.4 ± 0.30	1.3 ± 0.04	2.5 ± 0.09	2.1 ± 0.19
2	2.0 ± 0.03	1.6 ± 0.03	2.4 ± 0.10	2.5 ± 0.08
3	2.3 ± 0.06	1.6 ± 0.09	2.7 ± 0.04	2.3 ± 0.04
4	2.4 ± 0.14	1.6 ± 0.08	1.4 ± 0.06	1.8 ± 0.80
5	1.9 ± 0.13	1.1 ± 0.02	2.3 ± 0.17	1.7 ± 0.18
Mean	2.20 ± 0.11	1.44 ± 0.10[a]	2.26 ± 0.23	2.08 ± 0.15[b]

The experiment was repeated five times, and the mean scores for six duplicate samples from each experiment are shown, together with their standard errors (SEM). The effect of the antibody, in each experiment, was greater on animal than on vegetal cells. The mean scores from all six experiments were then compared using unpaired *t*-tests. The difference in aggregation is significant with respect to animal, but not vegetal cells.
A, Animal cell aggregation at 3 h, control IgM ± SEM; B, animal cell aggregation at 3 h, M4B ± SEM; C, vegetal cell aggregation at 3 h, control IgM ± SEM; D, vegetal cell aggregation at 3 h, M4B ± SEM.
[a]Unpaired *t*-tests, $P = 0.001$; [b]$P = 0.525$.

components of this complex may be glycoglycerolipids. These have previously been shown to be abundant in the gut, which would correlate well with our observation that at larval stages M4B antigen is almost completely confined to the gut. It remains possible that the carbohydrate epitope which is recognized by M4B may be common to a number of different glycolipid species. In addition, variation in fatty acid chain length in the lipid component is thought to produce glycolipid isoforms which migrate at different rates on HPTLC (Magnani *et al.*, 1987).

We have shown here that blastomeres isolated in calcium- and magnesium-free saline retain the capacity to reaggregate rapidly after treatment with trypsin in the presence of calcium, a technique designed to remove calcium-independent adhesion molecules and preserve calcium-dependent ones (Takeichi *et al.*, 1979). This result confirms the work of Nomura *et al.* (1986) which showed that cell−cell adhesion in the *Xenopus* blastula is calcium dependent. We demonstrate that the aggregation of blastomeres after this treatment is inhibited by M4B antibody. In addition, the reaggregation of dissociated but untrypsinized blastomeres is inhibited both by whole M4B IgM, and by univalent fragments derived from it. It is clear that the M4B antigen is therefore involved in the calcium-dependent adhesion of blastomeres to each other. There is good evidence from other species that carbohydrate antigens carried on glycolipids are involved in cell recognition and adhesion events. The SSEA-1/Lewis-X antigen has been shown to be involved in intercellular adhesion in early mouse embryos (Bird and Kimber, 1984) and F9 cells (Eggens *et al.*, 1989).

Even at concentrations of 100 μg/ml, M4B did not completely abolish blastomere reaggregation. This suggests that other calcium-dependent adhesion systems are at work. A novel cadherin has recently been identified at the blastula stage in *Xenopus* (Angres *et al.*, 1991; Ginsberg *et al.*, 1991), and antibodies against this cadherin partially block blastomere reaggregation in a similar way to M4B (Angres *et al.*, 1991). In early mouse embryos, both cadherin and glycolipid-mediated cell adhesion mechanisms are known to coexist (Hyafil *et al.*, 1980; Peyriéras *et al.*, 1983; Eggens *et al.*, 1989). In addition, in *Xenopus* embryos, there may be other surface carbohydrates involved, or other classes of adhesion proteins (e.g. integrins) which appear during the blastula stages. This allows a wide range of possible mechanisms of action of M4B. It may act homophilically, or it may bind to different molecules on the partner cell surface. These could be either different carbohydrates, or lectin-type proteins such as selectins. There is evidence that the SSEA-1/Le^X antigen can bind both to itself (Eggens *et al.*, 1989) and to selectin class proteins (Larsen *et al.*, 1990). Alternatively, M4B may act by modulating the distribution or conformation of other molecules on the surface of its own cell, e.g. cadherins. However, the reported distributions of M4B antigen and cadherin suggest that there is no obvious codistribution of the two molecules. We report here that the M4B antigen is differentially concentrated in the animal half of the blastula, whereas Angres *et al.* (1991) report that the blastula cadherin (U-cadherin, or E/P-cadherin) is evenly distributed throughout the blastula stage.

Perhaps the most interesting observation here is that M4B antigen is differentially expressed during the blastula stage, being more concentrated in the animal hemisphere. We have shown by direct experiments that this distribution correlates with the ability of the antibody to block aggregation, animal cells being most affected. In the absence of blocking antibody, animal and vegetal cells aggregate at the same rate. This experiment suggests that animal cells are more dependent for their aggregation on the M4B antigen than are vegetal cells, and also suggests that vegetal cells have additional unknown adhesion molecules. The most interesting question to be answered now is how these complex adhesive interactions control the onset of differential cell adhesion that occurs during the blastula stage and leads to tissue segregation during gastrulation.

Materials and methods

Embryological techniques

Adult *Xenopus laevis* females were stimulated to lay eggs by administering 800 IU chorionic gonadotrophin ('Chorulon'; Intervet) into the dorsal lymph sac. Eggs were fertilized artificially, then flooded with 0.1 × modified Barth's saline (HEPES) (0.1 × MBSH; Gurdon, 1977). Eggs and embyros were dejellied with 2% (w/v) L-cysteine−HCl (Sigma), pH 7.8, then washed extensively with 0.1 × MBSH. Embryos were staged according to Nieuwkoop and Faber (1967). Dissections were carried out in full strength MBSH on a base of 2% agarose. Vitelline membranes were removed with watchmaker's forceps and embryos were cut into fragments which were dissociated in 67 mM phosphate buffer. Dissociated cells were transferred by serum-coated mouth pipette into agarose-coated wells of 24-well tissue culture plates containing hybridoma supernatant or purified Ig diluted in MBSH. Control wells contained MBSH only, or control hybridoma medium diluted in MBSH, as appropriate. The cells were immediately swirled together into the centre of the wells to initiate aggregation.

Trypsinization of blastomeres

Dissociated blastomeres were immersed in calcium- and magnesium-free MBSH (CMBSH) containing 0.01% (w/v) trypsin (Sigma type I) for 20 min at room temperature. They were then transferred by serum-coated mouth pipette to 0.01% (w/v) soybean trypsin inhibitor (Sigma type I-S) in CMBSH for 5 min, washed 3 times for 5 min in CMBSH, and transferred to MBSH in aggregation assay plates as above.

Raising of monoclonal antibodies

Dejellied stage 9 embryos were homogenized in 1 mM EDTA, 1 mM PMSF, 0.01 mM leupeptin (all from Sigma) in phosphate-buffered saline (PBS). Yolk platelets and pigment granules were spun off at 2000 *g* for 3 min and the supernatant, excluding the uppermost layer of lipid was transferred to a Sorvall SS34 rotor and spun at 48 000 *g* for 15 min to give a crude membrane preparation in the pellet. This, or, in some instances, crude homogenate, was used to immunize female BALB/c mice by serial i.p. injections. Initial immunizations were in complete Freund's adjuvant (Difco), with subsequent injections in incomplete adjuvant. Fusion was carried out according to the method of Galfre *et al.* (1977) using mouse myeloma line X63.Ag8.653 (Flow Labs). Hybrids were selected in HAT medium and supernatants were screened by indirect immunofluorescence on ovary sections as described below.

Immunoglobulin preparation

Cell culture reagents were purchased from Northumbria Biologicals. Hybridoma lines were cloned by serial dilution and cultured in serum-free medium (SF-1) + 2 mM L-glutamine supplemented in early stages with ESG (Ewing sarcoma growth factor) and HECS (human endothelial culture supernatant) the manufacturer's recommended concentrations. Ig subclassing was by Ouchterlony immunodiffusion according to the method of Johnstone and Thorpe (1987). Ig was recoverd from culture supernatant by precipitation with polyethylene glycol (Mr = 8000; Sigma) essentially as described in Johnstone and Thorpe (1987). To separate Ig from BSA, the recovered protein was brought to 1 mg/ml in 10 mM Tris pH 8.5 and run onto a Waters DEAE 5PW anion exchange column, then eluted with a gradient of 0−400 mM NaCl in the same buffer using a Waters 600E HPLC unit. The flow rate was 1 ml/min, and 1 ml fractions were collected. Purified Ig was adjusted to 1 mg/ml in MBSH or PBS using Centricon 10 filtration units (Amicon). Protein determination was by Bio-Rad protein assay used according to the manufacturer's instructions with a bovine gamma globulin standard.

Preparation of univalent IgM fragments

Purified IgM at 1 mg/ml in PBS was digested with TPCK trypsin (Sigma), reduced, and alkylated according to the method of Matthew and Reichardt (1982). Samples from digests were run on 10% acrylamide SDS–PAGE gels according to the method of Laemmli (1970) under reducing and non-reducing conditions and subsequently stained with Coomassie brilliant blue (Sigma). Digests were size-fractionated on a Du Pont Zorbax GF 250 HPLC gel filtration column in MBSH at a flow rate of 0.5 ml/min. Confirmation of peak identities was by SDS–PAGE as above by reference to markers of relative mol. wt (Sigma SDS-6H).

Indirect immunofluorescence

Oocytes and embryos fixed in 2% (w/v) TCA were embedded in polyethylene glycol 400 distearate (Koch) containing 1% (w/w) acetyl alcohol and sectioned at 10 μm. Twelve and a half days post-coitum mouse embryos (MF1 strain) were fixed in 4% paraformaldehyde in PBS, embedded in gelatin and sectioned on a Bright cryostat. Living mouse embryonic stem cells were a gift from Martin Evans. Mounted sections were dewaxed in acetone and brought to PBS. Non-specific staining was blocked with 10% (v/v) goat or horse serum + 4% (w/v) BSA in PBS for 15 min at room temperature. Primary antibodies diluted in washing buffer [PBS + 1% (v/v) goat serum] was then applied for 1 h. Slides were washed 3 times for 5 min in washing buffer, then secondary antibody (FITC-conjugated goat anti-mouse Ig; Nordic) was applied for 1 h. Washing was repeated as above, except that 0.01% (w/v) eriochrome black was included in the last wash to quench yolk autofluorescence. Slides were mounted in 90% (v/v) glycerol containing 100 mg/ml DABCO [1,4-diazobicyclo(2,2,2)octane; Sigma] to suppress fluorescence bleaching. Sections were viewed with a Zeiss axiophot equipped for epifluorescence.

Crude lipid preparation

Staged embryos were homogenized in 3 vol of distilled water. Yolk and pigment were spun off at 2000 *g* for 4 min. Crude lipid was extracted from the supernatant in chloroform:methanol:water (5:10:3 v/v/v) according to the method of Magnani *et al.* (1987). The extract was dried down in a rotary evaporator at room temperature and resuspended in chloroform:methanol:water (60:35:4.5 v/v/v). The final lipid concentration approximated 400 embryos worth/ml.

High performance thin layer chromatography (HPTLC)

Aliquots (10 μl) of crude lipid extract were fractionated on aluminium-backed 5 × 7.5 cm HPTLC plates (0.2 mm Kieselgel 60 F254; Merck) in chloroform:methanol:0.02% $CaCl_2.2H_2O$ (60:40:9 v/v/v). After chromatography, plates were soaked in 0.1% (w/v) polyisobutyl methacrylate (Polysciences) in n-hexane and air-dried (Magnani *et al.*, 1987). For antibody staining, plates were sprayed with blocking buffer [PBS + 5% (w/v) non-fat milk powder ('Marvel') + 0.2% (v/v) Tween 20] and immersed in the same buffer for 15 min with gentle agitation. Plates were transferred to primary antibody diluted in blocking buffer for 2 h, washed 4 times for 20 min in the same buffer, and immersed for 2 h in secondary antibody (peroxidase-conjugated goat anti-mouse Ig; Tago) diluted 1:2500 in blocking buffer. Plates were washed 4 times for 20 min in blocking buffer and twice for 5 min in PBS, then processed for Amersham ECL chemiluminescence according to the manufacturer's instructions. Plates were exposed to Fuji X-ray film for 4 min.

Orcinol staining of HPTLC plates. Air-dried plates were sprayed with orcinol ferric chloride (Bial's reagent; Sigma), dried briefly, and placed at 100°C for 5–10 min to develop the stain.

Periodate treatment of HPTLC plates. Chromatograms were treated with polyisobutyl methacrylate as described above. They were then sprayed with 50 mM sodium acetate pH 4.5 and incubated for 1 h in the dark at room temperature in the same buffer containing 20 mM sodium-*meta*-periodate (Woodward *et al.*, 1985). Controls were exposed to buffer only. Plates were then transferred to 1% (w/v) glycine in PBS for 30 min at room temperature, rinsed in PBS, and processed for antibody staining as described above.

Sugar blocking

Oligosaccharides were made up as stock solutions of 4 mg/ml in PBS and diluted to 1 mg/ml in each antibody solution immediately before use.

Extraction of glycolipid and 'Folch' wash

Embryos were extracted by the method of Folch *et al.* (1957), as modified by Suzuki (1965). Five-hundred embryos (stage 9) were homogenized in 5 ml methanol to which 10 ml chloroform was then added. After further homogenization the extract was allowed to stand on ice for 4 h. Combined extracts were then mixed with 0.2 vol of 0.12 M NaCl and the mixture allowed to separate into two phases. The separated phases were then backwashed with appropriate quantities of freshly prepared lipid-free upper and lower phases. The original phases were combined with the two phases from the washings, the upper phase being extensively dialysed before being used for further analysis. Solvent in the lower phase was removed by rotary evaporation at 40°C in a Buchi rotavapor R110.

Silicic acid chromatography

Material in the lower phase (see above) was fractionated on a 1.4 cm diameter × 6.5 cm column of silicic acid (Sigma lot 90H0577 mesh size 325). The column was extensively washed with petroleum ether (60–80°C) and the lipid-free lower phase then applied in chloroform. The column was eluted with the same chloroform (10 column vol) followed by acetone (40 column vol) and then methanol (10 column vol). Each fraction was analysed by HPTLC following removal of solvent by rotary evaporation.

DEAE Sephadex A25

Material extracted into the upper phase (see above) was fractionated on DEAE Sephadex A25 (1.4 cm diameter × 6.5 cm, Sigma lot 40H0422) in acetate form, equilibrated with $CHCl_3$: $MeOH:H_2O$ (30:60:8 v/v/v). After elution with this solvent the column was washed with methanol and then finally eluted with 0.8 M sodium acetate in methanol (3 column vol).

Separation of glycolipids from phospholipid by Florisil chromatography

Material present in the methanol fraction from the silicic acid chromatography was subjected to mild acetylation conditions. Lipid present in this fraction was dissolved in acetic anhydride in pyridine (0.4 ml; 5:1 v/v) and left at room temperature overnight. The solvents were then removed in a stream of nitrogen with gentle warming, and the lipids dissolved in 1,2-dichloroethane (1 ml) and added to a column of acid-washed Florisil (1.1 cm diameter × 5.0 cm) in the same solvent. The column was eluted with 1,2-dichloroethane (10 ml) followed by 1,2-dichloroethane:acetone (80 ml, 1:1 v/v). Neutral lipids were eluted in 1,2-dichloroethane, followed by acetylated glycolipids in 1,2-dichloroethane:acetone (1:1 v/v) free of phospholipids which remain bound to the column. Acetylated glycolipid was deacetylated using 0.3 ml 0.5 M sodium methoxide in methanol (10 min at 50°C). Acetic acid (15 μl) was then added followed by chloroform (0.6 ml) and water 0.2 ml). After thorough shaking, the upper layer was removed and the lower layer washed twice more with methanol:water (1:1 v/v; 0.2 ml). Solvent was removed by rotary evaporation at 40°C and residual material analysed by HPTLC and staining with M4B.

Monosaccharide analysis

Samples of glycolipid previously purified by preparative size TLC plates were hydrolysed in 1 M HCl (1 ml) at 100°C for 12 h. On cooling, the hydrolysate was extracted three times with n-hexane (3 × 2 ml) and twice with chloroform (2 × 2 ml) and the aqueous material reduced to dryness under a stream of nitrogen at 35°C. Monosaccharides present in the hydrolysate were determined by the use of high-performance anion exchange chromatography under alkaline conditions. A Dionex (Sunnyvale, CA) BioLC system equipped with a PAD detector was used, and separation carried out using a Dionex CarboPac PA1 column eluted with 15 mM NaOH, followed by post-column addition of 0.3 M NaOH. Samples were introduced into a 25 μl sample loop, after filtration through a 0.2 μm filter (Anotop 1C).

Alkali-catalysed methanolysis

Purified glycolipid was treated according to Levine *et al.* (1975).

Glycerol estimation

A commercial kit (Boehringer Mannheim) was used on acid hydrolysates of TLC purified glycolipids according to manufacturers' instruction.

Acknowledgements

We thank Martin Evans for the kind gift of ES cells and Sue Kimber for the oligosaccharides. We thank Dr K.Nomura for his helpful comments, and Colin Sharpe for critical reading of the manuscript. We are grateful to the Wellcome Trust for financial support.

References

Angres,B., Müller,A.H.J., Kellerman,J. and Hausen,P. (1991) *Development*, **111**, 829–844.

Bird,J.M. and Kimber,S.J. (1984) *Dev. Biol.*, **104**, 449–460.

Brandley,B.K., Swiedler,S.J. and Robbins,P.W. (1990) *Cell*, **63**, 861–863.
Childs,R.A., Pennington,J., Uemura,K., Scudder,P., Goodfellow,P.N., Evans,M.J. and Feizi,T. (1983) *Biochem. J.*, **215**, 491–503.
Drickamer,K. (1988) *J. Biol. Chem.*, **263**, 9557–9560.
Eggens,I., Fenderson,B., Toyokuni,T., Dean,B., Stroud,M. and Hakomori,S.-I. (1989) *J. Biol. Chem.*, **264**, 9476–9484.
Feizi,T. (1991) *Trends Biochem. Sci.*, **16**, 84–86.
Fenderson,B.A., Eddy,E.M. and Hakomori,S.-I. (1990) *BioEssays*, **12**, 173–179.
Folch,J., Lees,M. and Stanley,G.H.S. (1957) *J. Biol. Chem.*, **226**, 497.
Francis,D., Toda,K., Merkl,R., Hatfield,T. and Gerisch,G. (1985) *EMBO J.*, **4**, 2525–2532.
Galfre,G., Howe,S.C., Milstein,C., Butcher,G.W. and Howard,J.C. (1977) *Nature*, **266**, 550–552.
Ginsberg,D., DeSimone,D. and Geiger,G. (1991) *Development*, **111**, 315–325.
Gooi,H.C., Feizi,T., Kapadia,A., Knowles,B.B., Solter,D. and Evans,M.J. (1981) *Nature*, **292**, 156–158.
Gurdon,J.B. (1977) *Methods Cell Biol.*, **16**, 125–139.
Heasman,J., Wylie,C.C., Hausen,P. and Smith,J.C. (1984) *Cell*, **37**, 185–194.
Hyafil,F., Morello,D., Babinet,C. and Jacob,F. (1980) *Cell*, **21**, 927–934.
Jessell,T.M., Hynes,M.A. and Dodd,J. (1990) *Annu. Rev. Neurosci.*, **13**, 227–255.
Johnstone,A., and Thorpe,R. (1987) *Immunochemistry in Practice*. 2nd edn. Blackwell Scientific, Oxford.
Kapadia,A., Feizi,T. and Evans,M.J. (1981) *Exp. Cell Res.*, **131**, 185–195.
Kintner,C.R. and Melton,D.A. (1987) *Development*, **99**, 311–325.
Künemund,V., Jungalwala,F.B., Fischer,G., Chou,D.K.H., Keilhauer,G. and Schachner,M. (1988) *J. Cell Biol.*, **106**, 213–223.
Laemmli,U.K. (1970) *Nature*, **227**, 680–685.
Larsen,E., Palabrica,T., Sajer,S., Gilbert,G.E., Wagner,D.D., Furie,B.C. and Furie,B. (1990) *Cell*, **63**, 467–474.
Levi,G., Crossin,K.L. and Edelman,G.M. (1987) *J. Cell Biol.*, **105**, 2359–2372.
Levine,M., Kornblatt,M.J. and Murray,R.K. (1975) *Can. J. Biochem*, **53**, 679–689.
Lloyd,C.W. (1975) *Biol. Rev.*, **50**, 325–350.
Magnani,J.L., Spitalnik,S.L. and Ginsburg,V. (1987) *Methods Enzymol.*, **138**, 195–207.
Matthew,W.D. and Reichardt,L.F. (1982) *J. Immunol. Methods*, **50**, 239–253.
Moscona,A.A. (1962) *J. Cell Comp. Physiol.*, **60 Suppl. 1**, 65–80.
Murray,R.K. and Narasimhan,R. (1990) In Kales,M. (ed.), *Handbook of Lipid Research*. Plenum Press, New York, volume 6, pp. 321–361.
Nieuwkoop,P.D. and Faber,J. (1967) (eds) *Normal Table of Xenopus laevis (Daudin)*. 2nd edn, North-Holland, Amsterdam.
Nomura,K., Uchida,M., Kageura,H., Shiokawa,K. and Yamana,K. (1986) *Dev. Growth Different*, **28**, 311–319.
Oppenheimer,S.B., Edidin,M., Orr,C.W. and Roseman,S. (1969) *Proc. Natl. Acad. Sci. USA*, **63**, 1395–1402.
Osborn,L. (1990) *Cell*, **62**, 3–6.
Peyriéras,N., Hyafil,F., Louvard,D., Ploegh,H.L. and Jacob,F. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 6274–6277.
Phillips,M.L., Nudelman,E., Gaeta,F.C.A., Perez,M., Singhal,A.K., Hakomori,S.-I. and Paulson,J.C. (1990) *Science*, **250**, 1130–1132.
Roberson,M.M. and Armstrong,P.B. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 3460–3463.
Schwarting,G.A., Jungalwala,F.B., Chou,D.K., Boyer,A.M. and Yamamoto,M. (1987) *Dev. Biol.*, **120**, 65–76.
Sharon,N. and Lis,H. (1989) *Science*, **246**, 227–234.
Solter,D. and Knowles,B.B. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 5565–5569.
Stoolman,L.M. (1989) *Cell*, **56**, 907–910.
Suzuki,K. (1965) *J. Neurochem.*, **12**, 629.
Takeichi,M., Ozaki,H.S., Tokunaga,K. and Okada,T.S. (1979) *Dev. Biol.*, **70**, 195–205.
Turner,A., Snape,A.M., Wylie,C.C. and Heasman,J. (1989) *J. Exp. Zool.*, **251**, 245–252.
Walz,G., Aruffo,A., Kolanus,W., Bevilacqua,M. and Seed,B. (1990) *Science*, **250**, 1132–1135.
Willison,K.R., Karol,R.A., Suzuki,A., Kundu,S.K. and Marcus,D.M. (1982) *J. Immunol.*, **129**, 603–609.
Woodward,M.P., Young,W.W.,Jr and Bloodgood,R.A. (1985) *J. Immunol. Methods*, **78**, 143–153.

Received on May 28, 1991; revised on July 31, 1992