High-molecular-weight glycoproteins are the major carriers of the carbohydrate differentiation antigens I, i and SSEA-1 of mouse teratocarcinoma cells

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The carriers of the carbohydrate differentiation antigens I, i and SSEA-1 were investigated in embryonal carcinoma cell lines of mouse and differentiated cell lines derived from them. Glycoproteins were studied by immunostaining ('Western blotting') of total cell lysates and immunoprecipitation from lysates of galactose oxidase/ NaB^3H_4 -labelled cells; glycolipids were investigated by immunostaining of thin layer chromatograms. The antigenic activities detected by immunofluorescence of cell smears were reflected in the antigenicities of high-molecular-weight glycoproteins. These were polydisperse and markedly susceptible to digestion with endo- β -galactosidase. Only the I antigen was detected on minor glycolipids. These observations indicate that glycoproteins rather than glycolipids are the major carriers of carbohydrate differentiation antigens I, i and SSEA-1 in the teratocarcinoma cell lines.

Studies with monoclonal antibodies have shown that a number of antigens which change during embryogenesis (termed stage specific embryonic antigens), or during the differentiation of embryonal carcinoma cells *in vitro*, are defined carbohydrate structures (Table 1). These include the Forssman antigen (structure 1), the I and i antigenic determinants (structures 2–5), SSEA-1 (structure 6) and FC 10.2 (structure 7). At later stages of differentiation and maturation these antigens may diminish, disappear or become restricted to certain tissues. The function of these carbohydrate structures is not yet known. One approach to investigate this is to determine the macromolecules that carry them. The carbohydrate structures are known to occur both on glycoproteins and glycolipids of a number of mammalian cell types (Feizi, 1981; Hounsell & Feizi, 1982; Hakomori, 1981*a,b*; Slomiany *et al.*, 1982; Kannagi *et al.*, 1982; Gooi *et al.*, 1983*a,b*); however, there is relatively little information on the macromolecules that carry them in embryos and teratocarcinoma cells. In the mouse embryonal carcinoma cell line F9, the Forssman antigen has been found associated with glycolipids (Willison *et al.*, 1982); and the human embryonic antigen FC 10.2 has been shown to occur on a 200 kDa

glycoprotein in the embryonal carcinoma cell line LICR LON HT39/7 (Williams *et al.*, 1982) and on a glycolipid in human meconium (Gooi *et al.*, 1983*b*). The carrier molecules for the Ii and SSEA-1 antigens of early mouse embryos and embryonal carcinoma cells have not, to our knowledge, been characterized, although in F9 cells SSEA-1 activity was found to be associated with macromolecules eluting from Bio-Gel columns as 500–1500 kDa aggregates (Andrews & Knowles, 1982).

In the present studies we have assessed the expression of the I, i and SSEA-1 antigens in undifferentiated and differentiated mouse teratocarcinoma cell lines by immunofluorescence and have investigated the carrier molecules of these antigens by (a) immunostaining of total solubilized glycoproteins after electrophoresis in SDS/polyacrylamide gels, (b) immune precipitation of surface glycoproteins and (c) immunostaining of glycolipid extracts after t.l.c. These studies have shown that the principal carriers of these carbohydrate antigens are polydisperse glycoproteins of high molecular weight (apparent molecular masses > 100 kDa).

Experimental

Cells

The following cell lines were studied: mouse embryonal carcinoma cell line F9 (Bernstine *et al.*,

Abbreviation used: SDS, sodium dodecyl sulphate.

1973) and MCP-6BRA (Goodfellow *et al.*, 1982) and parietal endoderm-type cell lines derived from them, F9-AC Cl 9, cited in the text as RF9 (Solter *et al.*, 1979) and MP-2 (Benham *et al.*, 1983), respectively [MCP-6BRA are hybrid cells resulting from the transfer of a human X-6 translocation chromosome (Goodfellow *et al.*, 1982) to the mouse embryonal carcinoma cell line PCC4 (Jakob *et al.*, 1973)]; mouse embryonal carcinoma cell line PSA-4 (Martin & Evans, 1975) and a visceral endoderm-type cell line PSA-5E derived from a parallel clone (PSA-5); undifferentiated PSMB cells and two day embryoid bodies derived from PSMB (Kapadia *et al.*, 1981). PSMB were maintained on mitomycin-inactivated feeder cells (STO mouse fibroblasts); induction of embryoid bodies was as described previously (Martin & Evans, 1975). The other cells were cultured as monolayers at 37°C in Dulbecco's minimal essential medium supplemented with 10% foetal calf serum, penicillin and streptomycin. Cells, with the exception of PSMB embryoid bodies, were detached with 0.2% EDTA in phosphate-buffered saline, pH 7.4. Contamination of PSMB cells by feeder cells was less than 1%. PSMB embryoid bodies were disaggregated with 0.25% trypsin ('trypsin 1:250'; Difco). Cell suspensions washed with phosphate-buffered saline were used for the preparation of cell smears and the immunostaining and immunoprecipitation experiments described below.

Antibodies

The monoclonal anti-I antibodies Ma and Step and anti-i antibody Den (Table 1) from patients with cold agglutinin disease (Feizi, 1981) and the mouse hybridoma antibodies anti-SSEA-1 raised against F9 cells (Solter & Knowles, 1978; Gooi *et al.*, 1981) and VEP8 and VEP9 raised against HL60 cell line (Rumpold *et al.*, 1982; Gooi *et al.*, 1983a) have been described previously. The anti-I and anti-i antibodies were used as plasma and the hybridoma antibodies as ascites fluid.

Immunofluorescence

Cell smears were prepared using a cytocentrifuge (Shandon, Runcorn, U.K.) and stored at -70°C in the presence of silica gel. Before immunofluorescence staining, cells were fixed in acetone at 4°C for 10 min. Indirect immunofluorescence was performed as described previously (Kapadia *et al.*, 1981). The binding of anti-I and anti-i antibodies (anti-I Ma, 1:100; anti-I Step, 1:700 and anti-i Den, 1:700) was detected using fluorescein-conjugated rabbit anti-human IgM (Dako, Copenhagen, Denmark) and the binding of the hybridoma antibodies anti-SSEA-1, VEP8 and VEP9 (1:100 dilutions) was detected with fluorescein-conjugated rabbit anti-mouse immunoglobulins (Nordic, Tilburg, The Netherlands).

Immunostaining of total cell lysates

Cells were solubilized in 2% SDS at 100°C for 5 min and stored at -70°C. Before electrophoresis, the following were added: 2-mercaptoethanol (3%); sucrose (5%) and Bromophenol Blue. SDS/polyacrylamide-gel electrophoresis was performed in 4–15% gradient slab gels using the conditions described by Laemmli (1970). After SDS/polyacrylamide-gel electrophoresis, one lane was removed for staining with 0.1% Coomassie Blue in methanol/acetic acid/water (5:1:4, by vol.) and the remainder of the gel was equilibrated for 30 min in 25 mM-Tris/192 mM-glycine buffer, pH 8.3. Electrotransfer of proteins from gels onto nitrocellulose sheets (Bio-Rad Laboratories) was performed at 4°C in Tris/glycine buffer for 2.5 h at 60 V. The nitrocellulose sheets were washed in 0.5 M-NaCl/20 mM-sodium phosphate buffer, pH 7.5 (solution A) containing 3% bovine serum albumin at 37°C for 60 min. The sheets were cut into strips and incubated with antibody or control serum diluted in solution A containing 3% bovine serum albumin at 4°C for 14 h. Antibody dilutions were: anti-i Den, 1:1000; anti-I Ma and Step and normal human plasma, 1:300; anti-SSEA-1, VEP8 and VEP9, and normal mouse serum, 1:100. Strips were washed twice at 4°C in solution A containing 1% Triton X-100 (30 min each) and once in solution A and incubated at 4°C for 3 h with either ¹²⁵I-labelled rabbit immunoglobulins to human μ chains or to mouse immunoglobulins. Rabbit immunoglobulins (Dako, Copenhagen, Denmark) were radiolabelled by the chloramine-T method (Greenwood *et al.*, 1963); specific radioactivity was 5 μ Ci/ μ g of protein and the antibodies were used at 2×10^6 c.p.m./ml of solution A containing 3% bovine serum albumin and 20% foetal calf serum. Strips were washed in solution A containing 1% Triton X-100 with a final rinse in solution A and air-dried. Autoradiography was performed using X-ray film obtained from Ceaverken AB, Strangras, Sweden.

Use of i- and I-active glycolipids as inhibitors of the immunostaining of F9 embryonal carcinoma cell glycoproteins with anti-i Den and anti-I Ma antibodies

The i-active glycolipid lacto-*N*-nor-hexaosylceramide (Niemann *et al.*, 1978) and the I-active ceramide deca-saccharide (Hanfland *et al.*, 1981) were tested as inhibitors of the immunostaining of F9 embryonal carcinoma cell glycoproteins with anti-i Den and anti-I Ma sera, respectively. These glycolipids were isolated from rabbit erythrocytes and were generously given by Dr. Peter Hanfland. For the inhibition assays each glycolipid was made into micelles with cholesterol and lecithin, as described previously (Feizi *et al.*, 1978). The final glycolipid concentration was 500 μ g/ml of solution A

containing 3% bovine serum albumin. This concentration of glycolipid was chosen as it was the minimum amount required for complete inhibition of binding of anti-i Den (1:1000) and anti-I Ma (1:300) to radiolabelled i and I-active reference glycoproteins, respectively, in a soluble phase, double-antibody radioimmunoassay (Wood *et al.*, 1979).

For the inhibition of immunostaining experiments, electrotransfer of six lanes of electrophoresed F9 cell lysate onto nitrocellulose was carried out as described above. Following washing with 3% bovine serum albumin, two lanes were removed for immunostaining with anti-i Den and anti-I Ma sera. The remaining lanes were air-dried and stored at -70°C . Using the autoradiographs of the immunostained lanes as templates, segments of the dried lanes were cut out and used for inhibition of immunostaining studies as follows: anti-i Den and anti-I Ma were pre-incubated at 4°C for 60 min with i-active and I-active glycolipids, respectively, and added to the segments of nitrocellulose at 4°C for 14 h. As a control, antibodies were preincubated with micelles of cholesterol and lecithin alone. After incubation these segments were washed and incubated with ^{125}I -labelled rabbit anti-IgM as described above.

Endo- β -galactosidase treatment of F9 embryonal carcinoma cell glycoproteins on nitrocellulose

Electrotransfer of the SDS lysate of F9 cells from a 4–15% polyacrylamide gradient slab gel onto nitrocellulose was performed as described above. After transfer, the nitrocellulose was washed with 3% bovine serum albumin as above and incubated at 40°C for 2 h with an endo- β -galactosidase purified from *B. fragilis* (specific activity 150 units/mg of protein) (Scudder *et al.*, 1983a). This enzyme cleaves internal GlcNAc(β 1-3)Gal(β 1-4/3)GlcNAc-(Glc) sequences and has a similar specificity to the previously reported endo- β -galactosidase (Fukuda *et al.*, 1978) from *E. freundii*. The enzyme was diluted (1 unit/ml) in a solution containing 375 mM-NaCl, 50 mM-sodium acetate buffer, pH 5.8, and 3% bovine serum albumin. The nitrocellulose was washed in solution A and immunostained as described above.

Immunoprecipitation of radiolabelled cell-surface glycoproteins

Detached cells were radiolabelled externally by the galactose oxidase/ NaB^3H_4 method (Gahmberg & Hakomori, 1973). Briefly, 1×10^8 cells per ml of Dulbecco's phosphate-buffered saline were treated with 0.2 units of neuraminidase/ml (from *Vibrio cholerae*, specific activity ≥ 20 units/mg of protein; Behringwerke AG, Marburg, Germany) and 10 units of D-galactose oxidase/ml (specific activity 714 units/mg; Kabi Vitrum, Stockholm, Sweden) at

37°C for 1 h. The cells were washed with 3×10 ml of phosphate-buffered saline and labelled with ^3H by the addition of 5 mCi of NaB^3H_4 , specific radioactivity 10 Ci/mmol (The Radiochemical Centre, Amersham, Bucks., U.K.). After 30 min at 18°C , the cells were washed with 6×10 ml of phosphate-buffered saline and solubilized in phosphate-buffered saline containing 1% zwitterionic detergent Empigen BB (Albright and Wilson, Whitehaven, U.K.), 2 mM-phenylmethanesulphonyl fluoride and 0.02% NaN_3 and centrifuged at 2000 g for 15 min to remove the unsolubilized nuclear pellet. No radioactivity was detected in the pellet. The lysates were stored at -70°C and recentrifuged in a Beckman Microfuge 152 for 5 min immediately before use. The supernatant, containing approx. 90% of the radioactivity of the 2000 g supernatant, was used in immune precipitation experiments as follows: 100 μl containing 1×10^6 – 3×10^6 c.p.m. were incubated at 4°C for 15 h with antisera or ascites fluids (20 μl of 1:10 dilution) followed by an excess of rabbit immunoglobulins against human μ chains or mouse immunoglobulins. Immune precipitates obtained by centrifugation at 2000 g for 20 min were washed and solubilized as described previously (Childs & Feizi, 1981). An aliquot was removed for determination of radioactivity and the remainder was analysed by SDS/polyacrylamide-gel electrophoresis as described above; radiolabelled bands were visualized by fluorography at -70°C (Bonner & Laskey, 1974). Radioactivity precipitated with control normal human serum or normal mouse serum was $\leq 2\%$ and with the antibodies up to 17% of the starting radioactivity.

Preparation of glycolipids

Lipids were extracted from 0.3–2.0 ml of packed cells with 20 vol. of chloroform/methanol (2:1, v/v) followed by chloroform/methanol/water (10:10:1 and 10:20:3, by vol.). The combined extracts were evaporated to dryness, dissolved in chloroform/methanol (2:1, v/v) and submitted to Folch partition by adding 0.25 vol of water (Folch *et al.*, 1957). The lower phase was repartitioned twice with chloroform/methanol/water (3:48:47, by vol.) and the resulting three upper phases were combined and evaporated to dryness. The lower phase was evaporated and the residue was peracetylated with pyridine/acetic anhydride (3:2, v/v). Peracetylated glycolipids were isolated as described by Saito & Hakomori (1971), combined with the upper phase lipids and deacetylated with 0.05 M-sodium methoxide in chloroform/methanol (1:2, v/v). The total glycolipids thus obtained were subjected to column chromatography on DEAE-Sephadex A-25 to separate neutral and acidic glycolipids according to the method of Yu & Ledeen (1972).

The cell residues after chloroform/methanol ex-

traction were dissolved in 1 M-NaOH and the protein content was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

T.l.c. and immunostaining of glycolipids

The binding of antibodies to glycolipids was detected as described by Magnani *et al.* (1981) with some modifications. Glycolipids were chromatographed on HP-TLC plates (aluminium sheets, silica gel 60; E. Merck) using as solvent systems, chloroform/methanol/water (11:9:2, by vol) for neutral glycolipids and chloroform/methanol/0.5% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in water (11:9:2, by vol.) for acidic

glycolipids. The chromatograms were treated with 5% bovine serum albumin in phosphate-buffered saline for 2 h and incubated at 4°C for 16 h with the following antibodies: anti-I Ma, anti-I Step and anti-i Den, (1:300 dilution); anti-SSEA-1 and VEP9, (1:100 dilution); and the rat monoclonal anti-Forssman antibody, M1/22 (Sera-Lab, Crawley Down, U.K., used without dilution). The plates were washed four times with phosphate-buffered saline, 30 min each, and incubated at 4°C for 4 h with ^{125}I -labelled rabbit antibodies (4×10^6 c.p.m./ml) against human IgM, mouse immunoglobulins or rat immunoglobulins as appropriate. Plates were washed six times with phosphate-buffered saline (20 min each) and subjected to autoradiography (14–40 h).

Fig. 1. Immunofluorescence staining of the I, i and SSEA-1 antigens on mouse embryonal carcinoma and differentiated cell lines

Fixed cell smears of embryonal carcinoma and differentiated cell line pairs F9/RF9, MCP-6/MP-2 and PSA-4/PSA-5E were stained with anti-I Ma, anti-I Step, anti-i Den and anti-SSEA-1 as described in the Experimental procedures. Magnification 165 $\times$.

Results

Immunofluorescence of the carbohydrate antigens *I*, *i* and SSEA-1 in embryonal carcinoma cell lines and in differentiated cell lines derived from them

By immunofluorescence, *I* and *i* antigenic determinants were found to be expressed to varying degrees in all the cell lines (Figs. 1 and 2). There were differences in the cell line pairs with respect to the fluorescence intensities and the proportions of cells showing immunofluorescence with the two anti-*I* antibodies Ma and Step, and anti-*i* Den. The

Fig. 2. Histograms showing the expression of *I*, *i* and SSEA-1 antigens on the mouse embryonal carcinoma and differentiated cell lines

The intensities of immunofluorescence are graded as follows: □, weak; ▨, moderate; ▩, strong; ■, very strong. Percentage of cells stained is shown on a logarithmic scale. Column 1 shows the staining reactions of the undifferentiated cell lines F9, MCP-6, PSA-4 and PSMB; column 2 shows the reactions of the corresponding differentiated cell lines RF9, MP-2, PSA-5E and PSMB embryo bodies. Abbreviations for antibodies used are: iD, anti-*i* Den; Im, anti-*I* Ma; Is, anti-*I* Step; S, anti-SSEA-1; V8, VEP8 and V9, VEP9.

I(Ma) antigenic determinant was expressed in the majority (greater than 80%) while *i* antigen was found only in sub-populations of cells in the eight embryonal carcinoma systems examined, ranging from 2% in RF9 and PSA-5E to 50% in MP-2. The finding of a sub-population of cells in the PSMB embryoid bodies with strong expression of the *i* antigen is in agreement with earlier observations (Kapadia *et al.*, 1981); these cells correspond to the primary endoderm of the early mouse embryo and are the first differentiated cells to appear in the embryoid bodies (Martin & Evans, 1975).

The positive immunofluorescence of F9 and MCP-6 cell lines with anti-SSEA-1 (Figs. 1 and 2) and the lack of staining of the parietal endoderm-type cell lines RF9 and MP-2 derived from them, confirms earlier observations (Solter *et al.*, 1979; Benham *et al.*, 1983). Similarly SSEA-1 was expressed in the PSA-4 cell line but not in the visceral endoderm-type PSA-5E cell line. Undifferentiated PSMB cells behaved like 1–4 cell stage mouse embryos in their lack of expression of SSEA-1, whereas 50% of cells in the differentiating embryoid bodies derived from them acquired this antigen. Immunofluorescence with the anti-human granulocyte antibodies VEP8 and VEP9 resembled that seen with anti-SSEA-1.

All of these antigens were visualized not only on the cell surface but also intracellularly, often in the form of perinuclear or granular juxtanuclear fluorescence.

The glycoprotein carriers of the *I*, *i* and SSEA-1 antigens

Immunostaining of total cell lysates, after electrophoresis in SDS/polyacrylamide gel and transfer onto nitrocellulose paper, showed that the determinants recognized by all the anti-carbohydrate antibodies used in this study were associated predominantly with glycoproteins of high molecular mass, in the region of 100 kDa and greater (Figs. 3–5). These glycoproteins migrated diffusely and a substantial proportion failed to enter the 4–15% polyacrylamide gradient gels. The immuno-staining largely corresponded to regions which gave little or no protein staining with Coomassie Blue (lane 1, panels A and C, Figs. 3–5) but were strongly labelled by galactose oxidase/NaB³H₄ treatment of intact cells (see below). There was reasonable accord in the relative intensities of immunofluorescence of the cytocentrifuge smears and the immunostaining of the high-molecular-weight regions of the cell lines with the various antibodies, with one exception, namely, anti-*I* Ma with the cell line pair PSA-4 and PSA5E. Although PSA-4 and PSA-5E cells showed similar immunofluorescence with anti-*I* Ma,

Fig. 3. Expression of the *I*, *i* SSEA-1, VEP8 and VEP9 antigens on glycoproteins of total lysates and ³H-labelled cell surface glycoproteins of F9 embryonal carcinoma cell line and the differentiated parietal endoderm-type cell line RF9 derived from it

Panels (a) and (c) are autoradiographs of the immunostaining of SDS lysates of F9 and RF9 cells respectively: 50 µg of cell protein (100 µg for Coomassie Blue staining, lane 1) was electrophoresed in SDS/polyacrylamide gels and electrophoretically transferred onto nitrocellulose and incubated with antiserum, immune ascites fluid or control serum followed by ¹²⁵I-labelled rabbit immunoglobulins to human IgM or to mouse immunoglobulins. Abbreviations are as follows: *i*, anti-*i* Den; *Im*, anti-*I* Ma; *Is*, anti-*I* Step; *Nh*, normal human serum; *S*, anti-SSEA-1; *V8*, VEP8; *V9*, VEP9; *Nm*, normal mouse serum. The two cell lines were tested together; exposure time of autoradiography was 2 days. Molecular weight markers were: myosin, 200 kDa; phosphorylase *a*, 94 kDa; bovine serum albumin, 68 kDa and ovalbumin, 43 kDa. Panels (b) and (d) are fluorographs of solubilized immune precipitates of galactose oxidase/NaB³H₄-labelled cell surface glycoproteins of F9 and RF9 cell lines, respectively. Lane 1, lysate of cells treated with neuraminidase and labelled by galactose oxidase/NaB³H₄; lane 2, lysate of cells treated with NaB³H₄ alone. Remaining lanes show immune precipitates with antibodies indicated above.

there was relatively little immunostaining of high-molecular-weight glycoproteins of the latter.

In agreement with the lack of immunofluorescence, SSEA-1 activity was lost or markedly reduced in the high-molecular-weight region of the differentiated cell lines RF9, MP-2 and PSA-5E (Figs. 3–5). This cannot be explained by a lack of high-molecular-weight glycoproteins in the differentiated cell lines since there was immunostaining of these glycoproteins with anti-*I* and/or anti-*i* antibodies. In fact, with the cell line MP-2 there was an increase in staining with the anti-*I*-antibodies relative to the undifferentiated MCP-6 cells (Fig. 5).

In addition to the diffuse high-molecular-weight glycoproteins a number of discrete bands also

reacted with anti-*I* Ma, anti-*i* Den, anti-SSEA-1 and the anti-granulocyte antibodies VEP8 and VEP9. With anti-*I* Ma and the mouse antibodies, these were numerous and they extended throughout the length of the gels but with anti-*i* Den there was staining of a single 54 kDa component. The majority of these bands did not differ markedly between the embryonal carcinoma and differentiated cell lines.

The ability of i and I-active glycolipids to inhibit immunostaining of F9 cells by anti-i Den and anti-I Ma

The reactions of anti-*I* Ma and anti-*i* Den with the diffuse high molecular weight glycoproteins and anti-*i* Den with the 54 kDa glycoprotein of F9 cells

Fig. 4. Expression of I, i and SSEA-1 antigens on glycoproteins of total lysates and ^3H -labelled cell surface glycoproteins of PSA-4 embryonal carcinoma cell line and the visceral endoderm-type cell line PSA-5E derived from it. Panels (a) and (c) are autoradiographs of immunostaining of SDS lysates of PSA-4 and PSA-5E cells, respectively. The procedures were as described in the legend to Fig. 3. Panels (b) and (d) are fluorographs of solubilized immune precipitates of galactose oxidase/ NaB^3H_4 -labelled cell surface glycoproteins of PSA-4 and PSA-5E cells, respectively. Abbreviations and molecular weight markers are as in Fig. 3.

could be specifically inhibited in the presence of I or i-active reference glycolipids (Fig. 6b). However, the reaction of anti-I Ma with the lower-molecular-weight components was not inhibited by the I-active glycolipid at a concentration of $500\text{ }\mu\text{g/ml}$. These observations raise the possibility that the reactions of these lower-molecular-weight components with anti-I Ma serum may be due to natural antibodies (Gooi & Feizi, 1982) unrelated to anti-I Ma. On the other hand, they may represent I-anti-I reactions of high affinity that are difficult to inhibit in the presence of the excess antibody used for immunostaining. The amount of glycolipid required to inhibit a 1:300 dilution of anti-I Ma was estimated in a soluble phase radioimmunoassay using a trace amount of radiolabelled, I-active, reference antigen. However, in a solid phase radioimmunoassay where the I-active, reference glycoprotein is bound to wells of plastic plates and may mimic more closely the situation of antigen bound to

the nitrocellulose strip, we have observed (J. Picard & T. Feizi, unpublished work) that the amounts of inhibitor required are substantially higher (10-fold or greater).

Specific inhibition studies were not performed with the mouse antibodies anti-SSEA-1, VEP8 and VEP9. However, in experiments using control serum and ascites fluid little or no immunostaining of the polydisperse high-molecular-weight glycoproteins was observed. With normal mouse serum some bands of lower molecular weights were weakly stained but these did not correspond to the discretely migrating bands seen with the hybridoma antibodies.

Susceptibility of the I, i and SSEA-1 active glycoproteins of F9 cells to digestion with endo- β -galactosidase

It was of interest to determine to what extent these antigens are expressed on carbohydrate chains of

Fig. 5. Expression of the I, i and SSEA-1 antigens on glycoproteins and ^3H -labelled cell surface glycoproteins of MCP-6 embryonal carcinoma cell line and the parietal endoderm-type cell line MP-2 derived from it

Panels (a) and (c) are autoradiographs of immunostaining of SDS lysates of MCP-6 and MP-2 cells, respectively. Procedures were as described in the legend to Fig. 3. Panels (b) and (d) are fluorographs of solubilized immune precipitates of galactose oxidase/ NaB^3H_4 labelled cell surface glycoproteins of MCP-6 and MP-2 cells, respectively. Abbreviations and molecular weight markers are as in Fig. 3.

poly-*N*-acetyl-lactosamine-type which are susceptible to digestion by endo- β -galactosidase. Treatment with this enzyme affected principally the immunostaining of high-molecular-weight glycoproteins (Fig. 6a). Their staining with anti-i and anti-SSEA-1 was abolished and there was a substantial reduction in the immunostaining with anti-I Ma. However, there was little effect on the expression of these antigens on the discretely migrating glycoproteins. On the contrary, the decrease in the I(Ma) staining of the diffuse glycoproteins revealed numerous restricted bands in the molecular weight region of 100–>200 kDa. These properties may be expected of I(Ma) determinants consisting of clustered short oligosaccharides, *O*-glycosidically linked to protein, as in structure 4a (Table 1). The I(Ma) activities of such oligosaccharides would be resistant to digestion with endo- β -galactosidase (Scudder *et al.*, 1983b).

Cell surface glycoprotein carriers of the I, i and SSEA-1 antigens

Galactose oxidase/ NaB^3H_4 treatment of neuraminidase-treated embryonal carcinoma and differentiated cell lines resulted in the labelling of high-molecular-weight glycoproteins (100–>200 kDa) in addition to components of lower molecular weights in the range 30–100 kDa (Figs. 3–5). Among these, high-molecular-weight glycoproteins in the range 70–>200 kDa were immune precipitated with the anti-I, i antibodies (Figs. 3–5, panels B and D). This was in agreement with the immunostaining of total cell lysates and indicated that at least a proportion of the Ii-active high-molecular-weight glycoproteins in the total cell lysates are associated with the cell surface. With the undifferentiated cells, the i- and I-active labelled glycoproteins migrated diffusely; however, with the three differentiated cell lines RF9, MP-2 and

Fig. 6. Immunostaining of glycoproteins of F9 embryonal carcinoma cell line following treatment with endo- β -galactosidase or in the presence of I and i-active glycolipids

(a) Nitrocellulose strips containing electrophoresed glycoproteins of F9 cells were incubated with either endo- β -galactosidase (E) or buffer control and immunostained with anti-i Den, anti-I Ma and anti-SSEA-1 as described in the Experimental procedures. Abbreviations and molecular weight markers are as described in the legend to Fig. 3. (b) Segments of the lanes of electrophoresed F9 cell glycoproteins on nitrocellulose were incubated with either anti-I Ma or anti-i Den in the presence of micelles containing the I or i-active glycolipid (GL), respectively, or with micelles without glycolipid as a control. The immunostaining was then as described in the Experimental procedures.

PSA-5E the surface glycoproteins precipitating with anti-I Ma resolved into two broad bands with molecular masses of approx. 130/230kDa (RF9), 160/270kDa (MP-2) and 190/330kDa (PSA-5E). Double bands were not visualized with the PSMB embryoid bodies (results not shown).

There was relatively little radioactivity in the immune precipitates with the hybridoma antibodies anti-SSEA-1, VEP8 and VEP9 and only weak polydisperse radioactive bands of high molecular weight were visualized with F9 and MCP-6 cells using anti-SSEA-1 (Figs. 3 and 5). This may be due to a decreased incorporation of ^3H into galactose residues of glycoproteins which are rich in the oligosaccharide, Gal(β 1-4)[Fuc(α 1-3)]GlcNAc(β 1-

The paucity of glycolipids carrying the Ii and SSEA-1 antigens

In contrast to the strong expression of these antigens on glycoproteins of teratocarcinoma cell

lines, there was little antigenic activity detectable on their glycolipids. Although the glycolipid extracts used for immunostaining were derived from 26–80-fold greater cell numbers than those used for glycoprotein analysis, there was no immunostaining with anti-i Den, anti-SSEA-1 and VEP9 and the only immunostaining detected was with anti-I Ma in the acidic glycolipids of RF9, MCP-6, MP-2 cells (Fig. 7) and in both the acidic and neutral fractions of PSA-4 cells, and with anti-I Step in the acidic fraction of MP-2 cells. As a positive control the monoclonal anti-Forssman antibody M1/22 was used with the neutral glycolipids of the F9/RF9 cell pair. As expected (Willison *et al.*, 1982), Forssman-active glycolipids were readily detected in the undifferentiated F9 cells.

Discussion

The immunofluorescence studies have revealed considerable heterogeneity in the expression of the I, i and SSEA-1 antigens, not only in the PSMB embryoid bodies which are known to be mixtures of undifferentiated embryonal carcinoma and primary endoderm cells, but also in the undifferentiated cell lines and the endoderm-type lines derived from them. The i antigen, which is largely associated with the primary endoderm of the early mouse embryo (Kapadia *et al.*, 1981), is expressed to varying degrees in 2–30% of the apparently undifferentiated embryonal carcinoma cells. These i-active cells presumably correspond to the sub-populations of spontaneously differentiating cells known to occur (Sherman & Miller, 1978; Kapadia *et al.*, 1981) in embryonal carcinoma cell lines. While none of the cell lines had immunofluorescence patterns identical with that of any specific cell type in the early mouse embryo, there were certain analogies with respect to their I, i and SSEA-1 antigenic activities. The majority of cells in the F9, MCP-6, PSA-4 lines and the PSMB embryoid bodies resemble embryo cells between the 16-cell and the early implantation stage; however, the majority of the cells of the PSMB cell line displayed no SSEA-1 antigen and resemble cells of the pre-8-cell mouse embryo (Kapadia *et al.*, 1981; Solter & Knowles, 1978). The differentiated cell lines RF9, MP-2 and PSA-5E have antigenic characteristics (lack of SSEA-1) of a later post-implantation stage. In their strong expression of the i antigen, cells of the PSMB embryoid bodies resemble the primary endoderm of the early mouse embryo.

The immunochemical studies in this report indicate that the glycoproteins rather than glycolipids are the major carriers of the I, i and SSEA-1 antigens of the teratocarcinoma cells examined. Although precise quantifications are difficult to

Table 1. Carbohydrate differentiation antigens of embryos and embryonal carcinoma cells recognized by monoclonal antibodies

References: (a) Willison & Stern, 1978; (b) Willison *et al.*, 1982; (c) Kapadia *et al.*, 1981; (d) Feizi *et al.*, 1982; (e) J. Pennington, unpublished work; (f) Gooi *et al.*, 1981; (g) Hounsell *et al.*, 1981; (h) Gooi *et al.*, 1983a; (i) Gooi *et al.*, 1983b.

Antibody	Oligosaccharide structure recognized	Designation	Expression on embryos and embryonal carcinomas	References
M1/22(anti-Forssman)	GalNAc(α 1-3)GalNAc(β 1-3)Gal	1	Mouse	<i>a, b</i>
anti-i Den	Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)GlcNAc(β 1-3)Gal	2	Mouse	<i>c</i>
anti-I*	Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)GlcNAc(β 1-3)Gal	3		
anti-I Ma	Gal(β 1-4)GlcNAc(β 1-3)Gal	4	Mouse and human	<i>c, d</i> <i>e</i>
	Gal(β 1-4)GlcNAc(β 1-3)GalNAc	4a		
Anti-I Step	GlcNAc(β 1-3)Gal(β 1-4)GlcNAc(β 1-3)Gal	5	Mouse	<i>c</i>
anti-SSEA-1 VEP8 and VEP9	Gal(β 1-4)GlcNAc(β 1-3)Gal Gal(β 1-4)GlcNAc Fuc(α 1-3)	6	Mouse	<i>f, g, h</i>
FC 10.2	Gal(β 1-3)GlcNAc(β 1-3)Gal(β 1-4)Glc	7	Human	<i>i</i>

* All anti-I antibodies react with the branched structure 3; anti-I antibodies from patients Ma and Step recognise different domains (structures 4 and 5) on this structure.

achieve, the 'Western blotting' technique and the chromatogram binding assay are of a comparable sensitivity. Only I-active glycolipids were detected in four of the six cell lines and it was necessary to use extracts from substantially larger numbers of cells than used for glycoprotein analysis. On the other hand, immunoreactive Forssman glycolipids were readily detected in the glycolipid extract from undifferentiated F9 cells. Furthermore, in studies which will be described elsewhere, we have detected glycolipids expressing the sialic acid-dependent antigen Pr₂, recognized by a human monoclonal antibody (Roelcke, 1974), in all of the acidic glycolipid extracts used in the present study. Using similar extraction procedures we have readily detected glycolipids with I and i activities in human erythrocytes and with SSEA-1 activities in the human promyelocytic cell line, HL60 (K. Uemura & T. Feizi, unpublished work). It will be of interest, therefore, to study mouse cells at later stages of differentiation in order to assess whether changes occur in the relative proportions of glycoproteins and glycolipids expressing various carbohydrate antigens.

Both the immunostaining of total cell lysates and the immune precipitation experiments have shown

that the main glycoprotein carriers of the I, i and SSEA-1 antigens in teratocarcinoma cells are of high molecular weight, polydisperse and susceptible to digestion by endo- β -galactosidase. Thus all three types of antigen are carried on carbohydrate chains of poly-N-acetyl-lactosamine type (Fukuda *et al.*, 1978). These glycoproteins almost certainly include the fucose-containing glycoproteins of embryos and embryonal carcinoma cells which express the antigens recognized by polyclonal anti-F9 sera (Muramatsu *et al.*, 1979; McCormick *et al.*, 1982). These are also susceptible to endo- β -galactosidase treatment (Muramatsu *et al.*, 1979) and yield high-molecular-weight glycopeptides upon Pronase digestion. Teratocarcinomas are known to produce glycosaminoglycans (Cantor *et al.*, 1976; Prehm, 1980; Rasilo & Renkonen, 1982). A proportion of these are keratan-sulphate-like (Rasilo & Renkonen, 1982) and susceptible to endo- β -galactosidase and might well have I, i-activities. In the differentiated cells, the high-molecular-weight double glycoprotein bands, labelled by galactose oxidase/NaB³H₄ and reactive with anti-I Ma, may include the basal lamina glycoproteins that are produced by teratocarcinoma-derived differentiated cells (Chung *et al.*, 1979; Hogan *et al.*, 1981; Howe

Fig. 7. Immunostaining of mouse teratocarcinoma cell glycolipids with anti-I antibodies

Neutral and acidic glycolipids isolated from teratocarcinoma cells were chromatographed on HP-TLC plates and immunostained with anti-I Ma (Im), or anti-I-Step (Is) as described in the Experimental procedures. Anti-Forssman (F) was used as a positive control. With each cell line pair lane 1 indicates undifferentiated and lane 2 the corresponding differentiated cells. Migration was upward. Panel (a) shows strong immunostaining of Forssman glycolipid from F9 cells with the monoclonal anti-Forssman antibody M1/22. Panel (b) shows immunostaining of a slow-migrating acidic glycolipid from RF9 cells with anti-I Ma. Panel (c) shows lack of immunostaining of the neutral glycolipids of MCP-6 and MP-2 cells with anti-I Ma and anti-I Step. Panel (d) shows faint staining (arrowed) with anti-I Ma of an acidic glycolipid migrating between G_{D1a} and G_{D1b} in MCP-6 and a slow migrating glycolipid in MP-2 cells, and staining with anti-I Step of a slow migrating acidic glycolipid in MP-2 cells. Panels (e) and (f) show immunostaining with anti-I Ma of a neutral glycolipid, migrating in the ceramide heptasaccharide region and a slow migrating acidic glycolipid from PSA-4 cells respectively. The glycolipids applied to the chromatography plates were extracted from samples containing protein (mg) as follows: F9 (1.3), RF9 (1.3), MCP-6 (4.0), MP-2 (2.3), PSA-4 (1.8) and PSA-5E (2.0). The chromatograms of cell glycolipids with reference standards were visualized with 0.2% orcinol in 2 M- H_2SO_4 (o). The following glycolipid standards were used: lane Fs, 0.1 μ g of Forssman glycolipid; lane A, a mixture of lactosylceramide (LacCer), neolactotetraosylceramide (paragloboside, PG), and ceramide hexasaccharide (VI, lacto-*N*-nor-hexaosylceramide), ceramide octasaccharide (VIII) and ceramide decasaccharide (X) from rabbit erythrocytes (obtained from Dr. P. Hanfland); lane B, a mixture of globotetraosylceramide (globoside, Glob), globopentaosylceramide (Forssman glycolipid, Fs) and gangliotetraosylceramide (asialo G_{M1} , As G_{M1}); lane C, human brain gangliosides. The nomenclature of Svennerholm is used for gangliosides (Svennerholm, 1963).

& Solter, 1980; Leivo *et al.*, 1982). We have previously noted strong immunofluorescence of I(Ma) antigen associated with the Reicherts membrane of PSMB embryoid bodies (Kapadia *et al.*, 1981). Recently, Cossu *et al.* (1983) have shown

that fibronectin from differentiating cells of a human teratocarcinoma cell line, but not from human fibroblasts, contains carbohydrate chains of the high-molecular-weight poly-*N*-acetyl-lactosamine type. These would be expected to express Ii-activi-

ties. In fact, we have identified fibronectin among Ii active glycoproteins isolated from human amniotic fluid using an anti-i absorbent column (R. A. Childs & T. Feizi, unpublished work).

High-molecular-weight glycoproteins are not confined to embryonic cells. For example, the 100kDa transport protein of erythrocytes known as band 3 expresses the I antigen (Childs *et al.*, 1978, 1979). The promyelocytic cell line HL60 contains diffusely migrating high-molecular-weight glycoproteins which resemble those of the teratocarcinoma cells in their immunostaining reactions with the anti-i and anti-I antibodies, anti-SSEA-1, VEP8 and VEP9 (R. A. Childs & T. Feizi, unpublished work). The monoclonal antibodies VEP8 and VEP9 were raised by immunization of mice with HL60 cells (Rumpold *et al.*, 1982) and it is intriguing that the specificities of these two antibodies (Gooi *et al.*, 1983a) are so closely related to that of anti-SSEA-1. The I and i antigens are expressed on a family of high-molecular-weight glycoproteins of human B lymphocytes (Childs & Feizi, 1981); among these are the differentiation molecules known as 'leucocyte common antigen' or T200 (Childs *et al.*, 1983). The corresponding glycoproteins on T lymphocytes have negligible amounts of Ii activities. Because they are such prominent structures on the cell surface and because of their different expression in lymphocytes that are functionally distinct, we have proposed (Childs *et al.*, 1983) that the I_i-active carbohydrate structures may have receptor functions that contribute to the differing properties of T and B lymphocytes.

Our studies have shown that, apart from high-molecular-weight glycoproteins, teratocarcinoma cells contain numerous discretely migrating proteins that react with anti-I Ma and anti-i Den sera, anti-SSEA-1, VEP8 and VEP9 immune ascites. The specificities of the majority of these reactions require further investigation. The reactivity of a 54kDa glycoprotein with anti-i Den could be inhibited in immunostaining experiments with a i-active glycolipid, although this glycoprotein was resistant to endo- β -galactosidase. The repeating poly-*N*-acetyl-lactosamine sequence (structure 2, Table 1) with i-activity is usually highly susceptible to digestion with this enzyme (Fukuda *et al.*, 1978); however certain modifications of the sequence GlcNAc(β 1-3)Gal(β 1- (e.g. sulphation of galactose or α 1-3 fucosylation of *N*-acetylglucosamine) confer resistance to this enzyme (reviewed by Scudder *et al.*, 1983a). It is possible, therefore, that the carbohydrate chains of the 54kDa glycoprotein contain a substitution which prevents the action of the enzyme without affecting the reaction with anti-i Den.

With monoclonal antibodies to defined carbohydrate structures it is possible to study with ease the carbohydrate moieties of the same proteins in

different cell types. This has recently been achieved with the high-molecular-weight glycoproteins of human T and B lymphocytes known as leucocyte common antigen (Childs & Feizi, 1981; Childs *et al.*, 1983). Thus the way is open for sequential studies of the carbohydrate antigens expressed on selected glycoproteins of embryonal carcinoma cells and their differentiated progeny. This would be a first step in investigating the contributions of carbohydrate structures to the functions of important glycoproteins such as the epidermal growth factor receptor and the insulin receptor, whose activities increase with the induction of differentiation (Rees *et al.*, 1979; Heath *et al.*, 1981).

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References

- Andrews, P. W. & Knowles, B. B. (1982) in *Teratocarcinoma and Embryonic Cell Interactions* (Muramatsu, T., Gachelin, G., Moscona, A. A. & Ikawa, Y., eds.), pp. 19–30, Japan Scientific Societies Press and Academic Press, Tokyo.
- Benham, F. J., Wiles, M. V., Banting, G., Andrews, P. & Goodfellow, P. N. (1983) in *Human Teratomas* (Damanjov, I., Knowles, B. B. & Solter, D., eds.), pp. 313–341, Humana Press, New Jersey.
- Bernstine, E. G., Hooper, M. L., Grandchamp, S. & Ephrussi, B. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3899–3903.
- Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88.
- Cantor, J., Shapiro, S. S. & Sherman, M. I. (1976) *Dev. Biol.* **50**, 367–377.
- Childs, R. A. & Feizi, T. (1981) *Biochem. Biophys. Res. Commun.* **102**, 1158–1164.
- Childs, R. A., Feizi, T., Fukuda, M. & Hakomori, S. (1978) *Biochem. J.* **173**, 333–336.
- Childs, R. A., Feizi, T. & Tonegawa, Y. (1979) *Biochem. J.* **181**, 533–538.
- Childs, R. A., Dalchau, R., Scudder, P., Hounsell, E. F., Fabre, J. W. & Feizi, T. (1983) *Biochem. Biophys. Res. Commun.* **110**, 424–431.
- Chung, A. E., Jaffe, R., Freeman, I. L., Vergnes, J.-P., Braginski, J. E. & Carlin, B. (1979) *Cell* **16**, 277–287.
- Cossu, G., Andrews, P. W. & Warren, L. (1983) *Biochem. Biophys. Res. Commun.* **111**, 952–957.
- Feizi, T. (1981) *Immunol. Commun.* **10**, 127–156.
- Feizi, T., Childs, R. A., Hakomori, S. & Powell, M. E. (1978) *Biochem. J.* **173**, 245–254.
- Feizi, T., Kapadia, A., Gooi, H. C. & Evans, M. J. (1982) in *Teratocarcinoma and Embryonic Cell Interactions* (Muramatsu, T., Gachelin, G., Moscona, A. A. & Ikawa, Y., eds.), pp. 201–215, Japan Scientific Societies Press and Academic Press, Tokyo.

- Folch, J., Lees, M. & Sloane Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497–509
- Fukuda, M. N., Watanabe, K. & Hakomori, S. (1978) *J. Biol. Chem.* **253**, 6814–6819
- Gahmberg, C. G. & Hakomori, S. (1973) *J. Biol. Chem.* **248**, 4311–4317
- Goodfellow, P. N., Banting, G., Trowsdale, J., Chambers, S. & Solomon, E. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 1190–1194
- Gooi, H. C. & Feizi, T. (1982) *Biochem. Biophys. Res. Commun.* **106**, 539–545
- Gooi, H. C., Feizi, T., Kapadia, A., Knowles, B. B., Solter, D. & Evans, M. J. (1981) *Nature (London)* **292**, 156–158
- Gooi, H. C., Thorpe, S. J., Hounsell, E. F., Rumpold, H., Kraft, D., Förster, O. & Feizi, T. (1983a) *Eur. J. Immunol.* **13**, 306–312
- Gooi, H. C., Williams, L. K., Uemura, K., Hounsell, E. F., McIlhinney, R. A. J. & Feizi, T. (1983b) *Mol. Immunol.* **20**, 607–613
- Greenwood, F. C., Hunter, W. M. & Glover, J. S. (1963) *Biochem. J.* **89**, 114–123
- Hakomori, S. (1981a) *Annu. Rev. Biochem.* **50**, 733–764
- Hakomori, S. (1981b) *Semin. Haematol.* **18**, 39–62
- Hanfland, P., Egge, H., Dabrowski, U., Kuhn, S., Roelcke, D. & Dabrowski, J. (1981) *Biochemistry* **20**, 5310–5319
- Heath, J., Bell, S. & Rees, A. R. (1981) *J. Cell Biol.* **91**, 293–297
- Hogan, B. L. M., Taylor, A. & Adamson, E. (1981) *Nature (London)* **291**, 235–237
- Hounsell, E. F. & Feizi, T. (1982) *Med. Biol.* **60**, 227–236
- Hounsell, E. F., Gooi, H. C. & Feizi, T. (1981) *FEBS Lett.* **131**, 279–282
- Howe, C. C. & Solter, D. (1980) *Dev. Biol.* **77**, 480–487
- Jakob, H., Boon, T., Gaillard, J., Nicholas, J. F. & Jacob, F. (1973) *Ann. Microbiol. (Paris)* **124**, 269–282
- Kannagi, R., Nudelman, E., Lavery, S. B. & Hakomori, S. (1982) *J. Biol. Chem.* **257**, 14865–14874
- Kapadia, A., Feizi, T. & Evans, M. J. (1981) *Exp. Cell Res.* **131**, 185–195
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Leivo, I., Alitalo, K., Risteli, L., Vaheri, A., Timpl, R. & Wartiovaara, J. (1982) *Exp. Cell Res.* **137**, 15–23
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Magnani, J. L., Brockhaus, M., Smith, D. F., Ginsburg, V., Blaszczyk, M., Mitchell, K. F., Steplewski, Z. & Koprowski, H. (1981) *Science* **212**, 55–56
- Martin, G. R. & Evans, M. J. (1975) *Cell* **6**, 467–474
- McCormick, P. J., Dimeo, A., Neuner, E. & Artzt, K. (1982) *Cell Diff.* **11**, 135–140
- Muramatsu, T., Gachelin, G., Damonville, M., Delarbre, C. & Jacob, F. (1979) *Cell* **18**, 183–191
- Niemann, H., Watanabe, K., Hakomori, S., Childs, R. A. & Feizi, T. (1978) *Biochem. Biophys. Res. Commun.* **81**, 1286–1293
- Prehm, P. (1980) *FEBS Lett.* **111**, 295–298
- Rasilo, M.-L. & Renkonen, O. (1982) *Eur. J. Biochem.* **123**, 397–405
- Rees, A. R., Adamson, E. D. & Graham, C. F. (1979) *Nature (London)* **281**, 309–311
- Roelcke, D. (1974) *Clin. Immunol. Immunopathol.* **2**, 266–280
- Rumpold, H., Obexerand, G. & Kraft, D. (1982) in *Natural Cell-Mediated Immunity* (Herberman, R. B., ed.), vol. 2, pp. 47–52, Academic Press, New York, London
- Saito, T. & Hakomori, S. (1971) *J. Lipid Res.* **12**, 257–259
- Scudder, P., Uemura, K., Dolby, J., Fukuda, M. N. & Feizi, T. (1983a) *Biochem. J.* **213**, 485–494
- Scudder, P., Uemura, K., Fukuda, M. N. & Feizi, T. (1983b) *Proc. Int. Glycoconjugate Symp. 7th*, 484–485
- Sherman, M. I. & Miller, R. A. (1978) *Dev. Biol.* **63**, 27–34
- Slomiany, B. L., Banas-Gruszka, Z., Zdebska, E. & Slomiany, A. (1982) *J. Biol. Chem.* **257**, 9561–9565
- Solter, D. & Knowles, B. B. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5565–5569
- Solter, D., Shevinsky, L., Knowles, B. B. & Strickland, S. (1979) *Dev. Biol.* **70**, 515–521
- Svennerholm, L. (1963) *J. Neurochem.* **10**, 613–623
- 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**, 785–793
- Willison, K. R., Karol, R. A., Suzuki, A., Kundu, S. K. & Marcus, D. M. (1982) *J. Immunol.* **129**, 603–609
- Wood, E. C., Lecomte, J., Childs, R. A. & Feizi, T. (1979) *Mol. Immunol.* **14**, 813–819
- Yu, R. K. & Ledeen, R. W. (1972) *J. Lipid Res.* **13**, 680–686