Blood-Group-Ii-Active Gangliosides of Human Erythrocyte Membranes

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More than ten new types of gangliosides, in addition to haematoside and sialosyl-paragloboside, were isolated from human erythrocyte membranes. These were separated by successive chromatographies on DEAE-Sephadex, on porous silica-gel columns and on thin-layer silica gel as acetylated compounds. Highly potent blood-group-Ii and moderate blood-group-H activities were demonstrated in some of the ganglioside fractions. The gangliosides incorporated into cholesterol/phosphatidylcholine liposomes stoichiometrically inhibited binding of anti-(blood-group I and i) antibodies to a radioiodinated blood-group-Ii-active glycoprotein. The fraction with the highest blood-group-I activity, I(g) fraction, behaved like sialosyl-deca- to -dodeca-glycosylceramides on t.l.c. Certain blood-group-I and most of the -i determinants were in partially or completely cryptic form and could be unmasked by sialidase treatment. Thus the I and i antigens, which are known to occur on internal structures of blood-group-ABH-active glycoproteins in secretions, also occur in the interior of the carbohydrate chains of erythrocyte gangliosides.

The blood-group-Ii antigens occur as isoantigens on erythrocytes (Race & Sanger, 1962) and as common cell-surface antigens on leucocytes (Lalezari & Murphy, 1967; Shumak *et al.*, 1971). They are also expressed on certain secreted glycoproteins, which behave as precursors or core structures of the blood-group-ABH antigens (Feizi *et al.*, 1971*a,b*; Feizi & Kabat, 1972). Blood-group-I and -i specificities are defined by a set of human erythrocyte cold agglutinins, which are often monoclonal immunoglobulin M proteins, and at least six types of blood-group-I and four types of blood-group-i antigenic determinant are recognized by the cold agglutinins of different patients (Feizi & Kabat, 1972). In attempts to define the precise sugar sequences that the various anti-(blood-group I and i) antibodies recognize, inhibition of precipitation assays were carried out by using oligosaccharides isolated from precursor-like glycoproteins (Feizi *et al.*, 1971*b*; Feizi & Kabat, 1972). The precipitation reaction of one anti-(blood-group I) antiserum (from patient Ma) could be inhibited by oligosaccharides containing the Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 6Gal structure, which constitutes the type-2 chain of blood-group precursors in secretions (Painter *et al.*, 1963). The reactions of several other anti-(blood-group I) and anti-(blood-group i) sera could not be inhibited, but the oligosaccharides were available in limited amounts and could not be used in high concentrations. Considerably less information is available on the chemical nature of the Ii antigens of human erythrocyte membranes. Certain glycoproteins found in the aqueous

phase of butanol extracts of erythrocyte membranes (Rosse & Lauf, 1970) or those prepared by biphasic extraction with aqueous chloroform/methanol (Hamaguchi & Cleve, 1972) and by aqueous-phenol extraction (Ebert *et al.*, 1975) have been reported to show blood-group-I activity. Other studies have indicated that blood-group-I activities are also carried on gangliosides (Yokoyama & Plocinik, 1965) and glycolipids (Anstee & Tanner, 1975). A glycolipid with ten carbohydrate units (Watanabe *et al.*, 1975) and others with 22–60 carbohydrate residues (polyglycosylceramides or macroglycolipids) (Gardas, 1976; Koscielak *et al.*, 1976) has been shown to carry blood-group-I in addition to blood-group-ABH activities. An anti-(blood-group i)-like cold agglutinin, from patient McC, has been reported to react with lacto-N-neotetraose or the glycosphingolipid termed paragloboside, both of which have the same carbohydrate structure, Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc (Tsai *et al.*, 1976). However, rabbit anti-paragloboside antibodies do not show blood-group-i specificity (Watanabe & Hakomori, 1976*a*; Schwarting & Marcus, 1977), and in the present study an extensively purified preparation of paragloboside did not appreciably inhibit the binding of antibody from patient McC to a blood-group-Ii-active glycoprotein.

We have found, however, that the binding of anti-(blood-group I and i) antibodies to a radioiodinated blood-group-Ii-active glycoprotein can be specifically inhibited by liposomes containing certain gangliosides. The fractions showing both blood-

group-I and -II activities have been characterized as a new type of ganglioside with 10–16 carbohydrate residues. The preparation, purification and properties of these blood-group-II-active gangliosides are described below.

Materials and Methods

Preparation of gangliosides

A total lipid extract of pooled blood-group-O cell membranes was prepared as previously described (Watanabe *et al.*, 1975; Hakomori & Watanabe, 1976), dissolved in chloroform/methanol (2:1, v/v) and partitioned with water by the method of Folch *et al.* (1957). The details of a general experimental procedure for preparation and characterization of blood-group glycolipids have been described elsewhere (Hakomori & Watanabe, 1976). The upper-phase glycolipids were dialysed against several changes of more than 20 vol. of water in Spectrapor 4000 dialysis tubing (Spectrum Medical Industries, Los Angeles, CA, U.S.A.) and freeze-dried. The yield of the upper-phase fraction was approx. 300 mg from 500 g of packed erythrocyte membranes. The upper-phase fraction, which contains long-chain neutral glycolipids, gangliosides, peptides and other unidentified material, was fractionated on a 2.5 cm × 56 cm column of DEAE-Sephadex A-25 (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) by elution with chloroform/methanol/water (30:65:8, by vol.) (solvent A), followed by gradient elution with methanol containing 0.03–0.35 M-ammonium acetate, by the modified method of Ando *et al.* (1976). The fraction eluted by solvent A represents neutral glycolipids containing relatively long carbohydrate chains (more than five carbohydrate units). Of the upper-phase fraction 65–70% comprised neutral glycolipids consisting of more than 20 kinds of molecular species, including those with blood-group-ABH and weak blood-group-I activities (Watanabe *et al.*, 1975; Hakomori & Watanabe, 1976), and 20–25% of the upper-phase fraction was composed of gangliosides (sialosylglycolipids). Three groups of gangliosides (I, II and III) were eluted with methanol containing approx. 0.03–0.07 M-, 0.10–0.12 M- and 0.20–0.27 M-ammonium acetate respectively (see Fig. 1a). Each group of gangliosides consisted of multiple components as revealed by t.l.c., and was further fractionated on a column (0.8 cm × 120 cm) of Iatro-beads, by gradient elution with a solvent system of chloroform/methanol/water by the method described by Momoi *et al.* (1976). Iatro-beads (GRS-8060) are a new porous silica-gel preparation (product of Iatron Co., 1-11-4 Higashi-kanda, Chiyoda-ku, Tokyo, Japan) of perfectly spherical beads of homogeneous diameter, which permits a rapid flow

even on a long column and gives good separation of gangliosides (Momoi *et al.*, 1976). Ganglioside groups I, II and III were thus fractionated into eight, I(a)–I(h), five, II(a)–II(e), and nine, III(a)–III(i), subfractions respectively that had distinctive t.l.c. patterns. Fraction I(g) was further separated into four components, I(g)1–I(g)4, by preparative t.l.c. as free gangliosides. To increase the recovery of gangliosides from silica-gel powder, the silica-gel suspension was treated with Dowex 50 (Price *et al.*, 1975).

In other experiments gangliosides were separated as acetylated derivatives; I(g) and I(h) resolved into six and seven fractions respectively, I(g)1Ac–I(g)6Ac and I(h)0Ac–I(h)6Ac. The conditions for separation of acetylated gangliosides were essentially the same as described previously for separation of blood-group glycolipids (Hakomori & Watanabe, 1976), but different solvent proportions were used in t.l.c. Gangliosides were acetylated in pyridine and acetic anhydride and the acetylated gangliosides were placed on a line 2 cm from the bottom of a pre-scored t.l.c. plate (silica-gel G), developed with the solvent 1,2-dichloroethane/acetone/water (20:30:1, by vol.) to the top of the plate (18 cm). The plates were dried and then developed with 1,2-dichloroethane/acetone/water (4:16:1, by vol.) up to 5 cm distance from the bottom in the same direction. The second development with more-polar solvents enabled previously immovable acetylated components to be separated into bands. After the second development, both edges of the plates were cut by cracking the plate, and developed by the orcinol/H₂SO₄ reaction and by the resorcinol/HCl reaction (Laine *et al.*, 1974). The bands on the intact middle part of the plates, corresponding to the spots revealed by orcinol or resorcinol reactions, were scraped and extracted with chloroform/methanol (1:1, v/v). The extraction was made by sonicating the suspension of silica gel in a sonic-cleaning bath (Bausch and Lomb, Rochester, NY, U.S.A.) for 5 min, followed by filtration through a sintered-glass filter with suction: the extraction procedure was repeated twice. The ganglioside acetates were deacetylated as described previously (Hakomori & Watanabe, 1976). Recovery of gangliosides as acetylated derivatives from silica gel after t.l.c. as above was much better than that of free gangliosides. There was no indication of degradation during these procedures applied to the monosialogangliosides, because resorcinol spots were always coincident with orcinol spots separated on t.l.c. The separation pattern of gangliosides as acetates was quite different from that as free gangliosides (see the Results section).

These ganglioside preparations were free from amino acids, peptides, neutral glycolipids and phospholipids as assessed by their reactions with sprays of ninhydrin, 5 M-H₂SO₄ (Laine *et al.*, 1974) and a

modified Zindzadze's reagent (Dittmer & Lester, 1964) after t.l.c.

The carbohydrate composition was analysed by g.l.c. on a SE-30 column by the modified method of Laine *et al.* (1972).

Preparation of paragloboside

Paragloboside was prepared from human erythrocytes by a method described previously (Siddiqui & Hakomori, 1973). Preparation (1) was isolated from the acid hydrolysate of sialosylparagloboside by chromatography on a Biosil A column (Bio-Rad, Richmond, CA, U.S.A.) by the method described by Watanabe & Hakomori (1976b). Preparations (2) and (3) were made from sialosylparagloboside by weak acid hydrolysis or sialidase treatment respectively.

Partial hydrolysis of gangliosides

Certain subfractions of I(g), [I(g)3Ac-I(g)5Ac] that showed strong blood-group-I activity by radioimmunoassay (see the immunological assay section) were pooled and subjected to mild acid hydrolysis or were treated with β -galactosidase (EC 3.2.1.23) and β -N-acetylhexosaminidase (EC 3.2.1.52) from jack beans (Li & Li, 1972) and neuraminidase from *Vibrio cholerae* (EC 3.2.1.18) (Behringwerke, Mannheim, Germany) as described previously (Hakomori *et al.*, 1971). (It was essential that the β -N-acetylhexosaminidase preparation used in this experiment was free from β -galactosidase activity. In a model experiment, paragloboside was not hydrolysed by the β -N-acetylhexosaminidase preparation, but was quantitatively hydrolysed and converted into GlcNAc β 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc $\rightarrow$ Cer by β -galactosidase under the conditions used in this experiment.) Since sodium deoxytaurocholate is necessary for hydrolysis of glycolipids, the same amount of deoxytaurocholate was included in a control experiment. About 20–30 μ g of glycolipids, made from a pool of the closely related I(g)3Ac-I(g)5Ac fractions, was dissolved in 20 μ l of 0.05 M-sodium citrate buffer, pH 4.5, that contained 20 μ g of sodium deoxytaurocholate in a small conical test tube. The mixture was sonicated in a sonic cleaning bath. Enzyme solution (5–10 μ l), which contained approx. 0.5 unit of one of the above enzymes, was mixed and incubated for 16 h. The same amount of enzyme was again added and further incubated for 16 h. For acid hydrolysis the glycolipids were dissolved in 50 μ l of water and sonicated briefly; 50 μ l of 0.1 M-HCl was added (final concentration of HCl 0.05 M) and the mixture was heated for 1 h at 80°C in Teflon-lined screw-capped micro-tubes (Kimble Products, Division of Owens-Illinois Inc., Toledo, OH, U.S.A.). After these treatments, each solution

was adjusted to pH 7 by adding 0.05 M-NH₃ and evaporated under an N₂ stream to complete dryness. The residue was extracted with 200–300 μ l of chloroform/methanol (2:1, v/v) and centrifuged at 800g for a few minutes, and the supernatant was evaporated to dryness.

Immunological analysis

Anti-(blood-group I) and anti-(blood-group i) sera from patients with chronic cold agglutinin disease were used for the determination of blood-group-I and -i activities by radioimmunoassay. Antisera were selected to include several types of blood-group-I and -i specificities (Feizi & Kabat, 1972). The anti-(blood-group I) sera selected have been described previously (Feizi & Kabat, 1972; Lecomte & Feizi, 1975); these were sera from patients Ma, Step, Gra, Da and Phi, which have been assigned to anti-(blood-group I) groups 1, 3, 4, 5 and 6 respectively, on the basis of their precipitation reactions with several blood-group substances. The anti-(blood-group i) sera studied were from patients McDon, Tho and Den (Feizi & Kabat, 1972) and a new serum from a patient Galli, provided by Dr. H. Rochant, Centre Hôpitalier Universitaire Henri Mondor, Créteil, France. In addition, the cold agglutinin from patient McC (Tsai *et al.*, 1976) with atypical anti-(blood-group i)-like properties was studied. Radioimmunoassays were carried out by a double-antibody method (Lecomte & Feizi, 1975), by using anti-(blood-group I and -i) sera, rabbit anti-(human immunoglobulin M) antiserum and a ¹²⁵I-labelled blood-group-II-active glycoprotein prepared from human meconium. The details of the method will be described elsewhere. Briefly, a glycoprotein-rich extract was prepared from a sample of human meconium by digestion with pepsin and precipitation with ethanol as described by Feizi *et al.* (1975). The extract was labelled with ¹²⁵I by the chloramine-T method (Greenwood *et al.*, 1963) and enriched for blood-group-I and -i activities by affinity chromatography on an adsorbent made with an anti-(blood-group I) antibody coupled to Sepharose 2B (Feizi *et al.*, 1975). Binding curves were obtained with the human anti-(blood-group I and i) sera and the labelled antigen by precipitation of the antigen-antibody complex with rabbit anti-(human immunoglobulin M) in the presence of human immunoglobulin M as carrier. Inhibition-of-binding assays were performed at antiserum dilutions that gave 25–30% binding of the radioiodinated reference antigen. Serial dilutions of the ganglioside fractions, complexed with cholesterol and phosphatidylcholine as carrier lipids, were used as inhibitors. The results were expressed as the amount of inhibitor required to give 50% inhibition of binding. As has been previously observed in complement fixation and haemagglutination assays with glycolipids (Marcus &

Schwartz, 1976), carrier lipids were essential for detection of antigenic activity in the ganglioside fractions by radioimmunoassay. The proportions of cholesterol, phosphatidylcholine and gangliosides were 2:2:1 (by wt.) respectively. These were mixed as solutions in chloroform/methanol (2:1, v/v), and evaporated to dryness under an N₂ stream. To incorporate the carrier lipids into the ganglioside fractions 0.9% NaCl was added to the dried material to achieve a concentration of gangliosides of 0.1–10 mg/ml. The resulting suspensions were heated to 50°C and sonicated at this temperature in a Dawe Sonicleaner (Dawe Instruments, Branson Electronics, CT, U.S.A.) for 5 min before testing.

The blood-group-I-active fraction I(g) and the pool of subfractions I(g)3Ac to I(g)5Ac were tested for blood-group-H, -M, -N and -T activities by haemagglutination-inhibition assays (Feizi *et al.*, 1971a). *Ulex europaeus* lectin (Dade Division, American Hospital Supply Corp., Miami, FL, U.S.A. and peanut lectin (Lothen *et al.*, 1975) from Dr. N. Sharon, Weizmann Institute of Science, Rehovoth, Israel, were used as anti-(blood-group H) and anti-(blood-group T) reagents respectively; rabbit antisera (Dade) were used as anti-(blood-group M) and anti-(blood-group N) reagents.

Results

Separation and partial characterization of erythrocyte gangliosides

The separation of the gangliosides into three groups by DEAE-Sephadex chromatography by using an ammonium acetate gradient can be easily correlated with their differing contents of sialic acid, whereas the separation by Iatro-beads depends on differences of carbohydrate chain links and branching.

The separation pattern of erythrocyte gangliosides on DEAE-Sephadex and of fraction I on Iatro-bead columns, as indicated by t.l.c., is presented in Fig. 1. The major ganglioside, which was previously identified as sialosylparagloboside, AcNeu2→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc→Cer (abbreviation: Cer, ceramide), is located in fraction I(c), which showed slightly higher mobility than brain GM₁ ganglioside in chloroform/methanol/water (60:35:8, by vol.). Haematoside (GM₃), the second major ganglioside, is located in fraction I(a). Fractions I(e) and I(f) are heterogeneous; the main component, which shows the same mobility as GD1a ganglioside of the brain, could be a sialosylgalactosylparagloboside as previously described (Watanabe *et al.*, 1974); the presence of GM₁ ganglioside (the same as brain GM₁ ganglioside) is also possible, but further elucidation of this fraction is necessary.

Fractions I(g) and I(h) consist of slow-migrating components. The I(g) fractions can be separated by

t.l.c. into components I(g)1, I(g)2, I(g)3 and I(g)4. These fractions were extracted from thin-layer silica gel. The ganglioside fraction I(g)1 gave a single band on t.l.c. in the chloroform/methanol system. By comparison with the migration of standard gangliosides and long-chain neutral glycolipids such as H₃ and Ac glycolipids, component I(g)1 could be a monosialosyl-deca- to dodeca-glycosylceramide and component I(g)2 monosialosyl-hepta- to -octa-glycosylceramide. However, I(g)2 fraction was still not homogeneous. The ganglioside fractions separated by the acetylation procedure [I(g)3Ac, I(g)4Ac and I(g)5Ac] as described in the Materials and Methods section had, after deacetylation, migrated on t.l.c. identically with I(g)1 fraction; thus fractions I(g)3Ac–I(g)5Ac were not distinguishable as free glycolipids on t.l.c.

Fractions I(h)0Ac–I(h)6Ac were an extreme minority, and after deacetylation they migrated as a band slower than fraction I(g)1 on t.l.c. It is assumed that they were monosialosyl derivatives with around 10–16 carbohydrate residues, namely they could be the sialosyl derivatives similar to H₄- or A^d-glycolipid (Hakomori & Watanabe, 1976; Watanabe *et al.*, 1975).

The carbohydrate analysis of I(g)1 and I(h) fractions indicated that they are composed of galactose, glucosamine, glucose, sialic acid and fucose bound to ceramide. The proportions of galactose: N-acetylglucosamine:glucose:sialic acid:fucose in I(g)1 and I(h) fractions were 5.5:4.5:1:1.5:0.3 and 6.3:5.2:1:1.6:0.2 respectively; these values were calculated based on glucose as 1.0. These analytical data also indicated that the fractions are a heterogeneous highly complex new type of ganglioside.

Radioimmunoassays of blood-group-I and -i activities of the ganglioside fractions and paragloboside

By radioimmunoassay little or no blood-group-Ii activities were detected at concentrations up to 1 mg/ml in the unfractionated neutral glycolipids and in the crude ganglioside fractions I and II eluted from the DEAE-Sephadex column; fraction III was active with the anti-(blood-group I) sera from patients Step and Gra (gave 50% inhibition at less than 100 μg/ml). The inhibitory activities of the ganglioside subfractions are shown in Table 1. The highest blood-group-I activities with antisera Step, Phi, Gra and Da were demonstrated in fractions I(f), I(g) and I(h); the same fractions also had blood-group-i activity of the McDon type. As little as 3–20 μg of these fractions/ml showed 50% inhibition of precipitation. None of these fractions had detectable activities with the anti-(blood-group I) serum from patient Ma and anti-(blood-group i) sera from patients Galli and McC.

Fig. 1. Elution pattern of (a) erythrocyte gangliosides from DEAE-Sephadex and (b) fraction I gangliosides on Iatro-beads column

In (a) 560mg of human blood-group-O erythrocyte gangliosides was separated on a DEAE-Sephadex column (2.5cm×56cm) with a gradient of ammonium acetate in methanol as shown in the ordinate. The gradient elution was performed by a three-chamber mixing system as described by Momoi *et al.* (1976). Each chamber contained 1 litre of 0.03M-, 0.09M- and 0.27M-ammonium acetate in methanol respectively. Fractions (20ml) were collected and each fraction was analysed by t.l.c. with resorcinol stain. Distribution of ganglioside spots as separated on t.l.c. is shown: the arrowed spots (GM₃, GM₁) indicate the positions of GM₃ and GM₁ gangliosides. The intensity of spots is indicated as follows: most intense, filled circle; less intense, open circle; less intense, speckled circle; least intense, broken circle. I, II and III indicate the fractions pooled and applied to an Iatro-beads column. Fraction I represents monosialogangliosides. In (b) 220mg of fraction I separated by DEAE-Sephadex was further separated on an Iatro-beads column (1.4cm×115cm) with a two-chamber gradient system. The first chamber contained 790ml of chloroform/methanol/water (30:20:1, by vol.) and the second contained 930ml of chloroform/methanol/water (15:35:2, by vol.). Fractions (20ml) were collected and examined by t.l.c. and resorcinol stain. Samples of volume 15μl were used on t.l.c., and therefore minor components present in each fraction were not revealed. The ordinate indicates the composition of the eluate; up to about 1700ml gradient elution was used; then the column was eluted with 90% methanol. Other symbols are as in (a).

The activities of Ig and Ih subfractions with various anti-(blood-group I) sera and with the anti-(blood-group i) serum from patient McDon are shown in Table 2. The differences in the antigenic activities of these fractions with anti-(blood-group i) serum from the patient McDon suggest that there may be structural differences in their carbohydrate moiety. The fractions obtained by the acetylation procedure

were as active as those obtained through procedures without acetylation. There were considerable differences in the expression of the various I and i (McDon) determinants among the I(h)Ac fractions (Table 2). The I determinant of Step type was almost equally expressed in fractions I(h)0Ac-I(h)4Ac, whereas determinants of the Phi and Gra types were not detectable in fraction I(h)1Ac. Fraction

Table 1. *I-* and *i*-antigen activities of erythrocyte ganglioside fractions determined by inhibition of binding of a ¹²⁵I-labelled blood-group-Ii-active glycoprotein to anti-(blood-group I) and anti-(blood-group i) sera

The inhibition-of-binding assays were carried out at dilutions of anti-(blood-group I and i) sera that gave 25–30% binding of the radioiodinated blood-group-Ii-active glycoprotein. Serial dilutions of the ganglioside fractions complexed with carrier lipids (see the Materials and Methods section) were used as inhibitors. The results were expressed as the concentration of inhibitor required to give 50% inhibition of binding. The fractions were tested at maximum concentrations of 500 µg/ml or 1 mg/ml. Only the fractions showing some inhibitory activity are tabulated. +, Active, but less than 50% inhibition at the maximum concentration tested; –, no activity at maximum concentration tested.

Ganglioside fractions	Sera ... Patient ...	Minimum ganglioside concentrations giving 50% inhibition (µg/ml)							
		Anti-(blood-group I)					Anti-(blood-group i)		
		Step	Phi	Gra	Da	Ma	McDon	McC	Galli
I(f)		25	+	+	+	–	+	–	–
I(g)		4	60	50	+	–	12	–	–
I(h)		3	15	12	+	–	+	–	–
II(d)		20	–	+	–	–	–	–	–
II(e)		+	–	+	–	–	–	–	–
II(f)		–	–	+	–	–	–	–	–
III(f)		80	–	+	–	–	+	–	–
III(g)		80	–	+	–	–	–	–	–
III(h)		+	–	+	–	–	–	–	–
III(i)		–	–	+	–	–	–	–	–

Table 2. *I-* and *i*-antigen activities of subfractions of I(g) and I(h) gangliosides

Inhibition-of-binding assays were carried out as described in Table 1 by using as inhibitors subfractions of the I(g) and I(h) gangliosides. The subfractions were obtained by preparative t.l.c. of these fractions in the free state and after acetylation (Ac fractions). The I(g)Ac and I(h)Ac fractions were deacylated before being tested (see the Materials and Methods section). The inhibitory activities of unlabelled Ii-active reference glycoprotein and of paragloboside are also shown. Maximum concentrations of inhibitors used were 100 µg/ml with sera from patients Step, Gra and McDon, and 30 µg/ml with sera from patients Phi, Da, Ma, McC and Galli. Abbreviations: +, active, but less than 50% inhibition at 30 or 100 µg/ml; –, not active at maximum concentration tested; nt, not tested; min, minimal activity (20% inhibition at 500 µg/ml). Preparations 2 and 3 were not tested with the other anti-(blood-group I and i) sera.

Fraction	Sera ... Patient ...	Concentrations (µg/ml) giving 50% inhibition							
		Anti-(blood-group I)					Anti-(blood-group i)		
		Step	Phi	Gra	Da	Ma	McDon	McC	Galli
Ii glycoprotein standard		3	4	7	40	8	3	3	2
I(g)		5	60	50	+	–	+	–	–
I(g) subfractions:									
1		17	nt	40	–	–	–	–	–
2		12	nt	–	+	–	10	–	–
3		–	nt	–	–	–	–	–	–
4		–	nt	–	–	–	+	–	–
I(g)Ac fractions:									
1		30	–	–	–	–	60	–	–
2		10	8	30	+	–	+	–	–
3		9	10	36	+	–	+	–	–
4		4	5	14	+	–	12	–	–
5		3	7	22	+	–	80	–	–
6		–	–	–	–	–	–	–	–
I(h)		2	15	12	+	–	+	–	–
I(h)Ac fractions:									
0		6	+	+	+	–	+	–	–
1		3	–	–	+	–	+	–	–
2		5	6	16	30	–	80	–	–
3		5	10	23	+	–	+	–	–
4		4	30	42	+	–	–	–	–
5		–	–	–	–	–	–	–	–
6		–	–	+	–	–	–	–	–
Paragloboside 1		–	–	–	–	–	–	–	–
2								min	
3								min

Fig. 2. *I*- and *i*-antigen activities of the pooled *I*(g)3Ac-*I*(g)5Ac fractions before treatment (○) and after treatment with β -galactosidase (EC 3.2.1.23) (▲), β -*N*-acetylhexosaminidase (EC 3.2.1.52) (▼), neuraminidase (EC 3.2.1.18) (■), and HCl (●). They were complexed to carrier lipids and tested as inhibitors of the binding of 10 μ l of the diluted anti-(blood-group *I* and *i*) sera to a 125 I-labelled blood-group-*Ii*-active glycoprotein. The specificity, source of serum and dilution are:

(a)	<i>I</i>	Step	1:4000
(b)	<i>I</i>	Phi	1:600
(c)	<i>I</i>	Gra	1:200
(d)	<i>I</i>	Da	1:200
(e)	<i>i</i>	McDon	1:40 000
(f)	<i>I</i>	Ma	1:4000
(g)	<i>i</i>	McC	1:25 000
(h)	<i>i</i>	Tho	1:4000
(i)	<i>i</i>	Den	1:60 000

I(h)4Ac had *I*-antigen activities, but it was not active with anti-(blood-group *i*) serum from patient McDon.

Since it was reported (Tsai *et al.*, 1976) that paragloboside (lacto-*N*-neotetraosylceramide) inhibited the haemagglutination of the anti-(blood-group *i*)-like cold agglutinin from patient McC, inhibition of binding studies were also carried out with this glycolipid. As in Table 2, the activity of three samples of paragloboside were negligible compared with some of the ganglioside fractions. As much as 500 μ g of paragloboside/ml hardly inhibited antibody from patient McC (Table 2), whereas as little as 5 μ g of acid-treated or neuraminid-

ase-treated *I*(g)Ac3-*I*(g)Ac5 gangliosides/ml gave 50% inhibition (see Fig. 2).

Blood-group-I and -i activities of partially hydrolysed gangliosides

The activities of the pooled *I*(g)Ac fractions with sera from patients Step, Phi and Gra were substantially diminished by treatment with β -galactosidase and slightly diminished by β -*N*-acetylhexosaminidase (Fig. 2). Removal of sialic acid by weak acid hydrolysis or treatment with neuraminidase did not cause a marked change in activity. The *I*-antigen activity with serum from patient Da and

the i-antigen activity with serum from patient McDon were completely lost after treatment with β -galactosidase, much diminished after treatment with β -N-acetylhexosaminidase, but notably enhanced after acid and neuraminidase treatment. I-antigen activity with serum from patient Ma and i-antigen activity with sera from patients Galli and McC were not detectable in the intact gangliosides, but activities with Ma and McC sera appeared after the I(g)Ac fractions were hydrolysed by weak acid or neuraminidase. These fractions were tested with two additional anti-(blood-group i) sera from patients Tho and Den; i-antigen activity with serum Tho was revealed by acid and neuraminidase treatment and with serum Den only after acid treatment. No activity with the anti-(blood-group i) serum Galli was detected in these degraded fractions. Under the conditions described, no fucosyl residues, but only sialosyl residues, would be hydrolysed (Stellner *et al.*, 1973), therefore the activities with the patient Ma, McC, Tho and Den antibodies were probably masked by sialosyl residues.

Other blood-group activities on the I(g) fractions

By haemagglutination-inhibition assays (Feizi *et al.*, 1971a) fraction I(g) and the I(g)3Ac-I(g)5Ac pool showed blood-group-H activity at 25 μ g/ml, but no blood-group-M, -N or -T activities at 50 μ g/ml. There was no change in these activities after treatment with acid and the three exoglycosidases.

Discussion

The present studies indicate that the ganglioside pattern of human erythrocytes is far more complex than was hitherto realized. Haematoside (AcNeu2 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc1 $\rightarrow$ 1Cer) (Marcus *et al.*, 1976; Hakomori & Strycharz, 1968), sialosylparagloboside (AcNeu2 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc1 $\rightarrow$ 1Cer) (Ando *et al.*, 1973; Siddiqui & Hakomori, 1973; Wherrett, 1973; Marcus *et al.*, 1976), sialosyl-galactosylparagloboside (AcNeu2 $\rightarrow$ 3Gal β 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc $\rightarrow$ Cer) (Watanabe *et al.*, 1974) and GM₁ ganglioside (the same as brain GM₁ ganglioside) (Marcus *et al.*, 1976) are the known gangliosides of human erythrocyte membrane. The presence of other minority gangliosides was previously noted, but they were not characterized (Watanabe *et al.*, 1974). The sequential chromatography on DEAE-Sephadex and Iatro-beads now reveals the presence of at least ten additional gangliosides, whose structures remain to be elucidated. It is apparent from the present study that a new type of ganglioside represented by fractions I(g) and I(h) carries Ii-antigen activities with a variety of specificities. The occurrence of blood-group-ABH determinants on multiple carrier molecules has been well established in the glycosyl-

ceramide series (Hakomori *et al.*, 1972; Stellner *et al.*, 1973; Koscielak *et al.*, 1973; Watanabe *et al.*, 1975). It is possible that I and i determinants are also carried on multiple glycosylceramides.

Assays of Ii-antigen activities of cellular glycolipids present considerable problems. First, in haemagglutination-inhibition assays glycosphingolipids or liposomes containing them when incubated with erythrocytes, may sometimes be incorporated into the membranes and obscure their inhibitory activity. Secondly, in double-diffusion tests in agar gel immunoglobulin M proteins have often shown non-specific precipitation, and the distinction between specific and non-specific reactions has been difficult; moreover, large amounts (100–200 μ g) of glycolipids are usually required in precipitation tests. Thirdly, the complement-fixation reaction is highly sensitive with glycolipid-containing liposomes in general; however, their reactions with anti-(blood-group Ii) sera are limited because Ii antigen-antibody complexes usually dissociate at 37°C (the temperature required for haemolytic indicator in the complement-fixation method). These difficulties have been eliminated in the radioimmunoassay system in which the binding of anti-(blood-group I and i) antibodies to a radioiodinated blood-group-Ii-active glycoprotein was successfully inhibited by liposomes consisting of glycosphingolipids, cholesterol and phosphatidylcholine. A stoichiometric inhibition of blood-group Ii antigen-antibody interactions by certain glycosphingolipid-containing liposomes has been demonstrated. These results clearly show that I and i determinants are carried on a subpopulation of the gangliosides of adult human erythrocytes. The determinants recognized by anti-(blood-group I) sera from patients Step, Phi and Gra are fully expressed on these gangliosides, whereas those reactive with the anti-(blood-group I) sera from patients Da and Ma and with all of the anti-(blood-group i) sera are partially or wholly masked, probably by sialosyl residues. In the present studies, I-antigen activities could not be demonstrated in the crude ganglioside fractions I and II although some of their purified subfractions were subsequently shown to be active. Lipid haptens may be masked by neighbouring glycolipids in liposomes. Such masking of blood-group-H-active glycolipids by globoside has been described (Stellner *et al.*, 1973) and provided an explanation for an earlier claim that blood-group-H-active glycolipids were absent from the glycolipid fraction of erythrocytes.

In contrast with a claim that paragloboside reacts strongly with the anti-(blood-group i)-like cold agglutinin from patient McC (Tsai *et al.*, 1976), the activity of extensively purified paragloboside, determined by radioimmunoassay, was negligible. On the other hand, some gangliosides, after removal of sialosyl residues by either acid or neuraminidase,

showed strong activity with this cold agglutinin. The discrepancy between the two studies cannot be explained readily. Rabbit anti-paragloboside antibodies so not show anti-(blood-group i) properties, namely they do not distinguish between cord blood and adult erythrocytes (Watanabe & Hakomori, 1976a; Schwarting & Marcus, 1977). It may be that the structure of paragloboside is not perfectly complementary to McC antibody, but may represent a part of the determinant recognized by this antibody, as suggested by Schwarting & Marcus (1977). Another possibility is that the paragloboside used in the study by Tsai *et al.* (1976) was contaminated with another highly active glycolipid.

The radioimmunoassays with the partially hydrolysed gangliosides indicate that the specificities of anti-(blood-group I) serum from patients Step, Phi, Gra and anti-(blood-group i) from patient McDon primarily involve terminal β -galactosidic structures. The effect of β -N-acetylglucosaminidase raises the possibility that β -N-acetylhexosaminyl termini are also involved in the full expression of the I determinants. Thus the determinants may consist of branched carbohydrate chains, with terminal galactosyl and terminal β -N-acetylhexosaminyl residues. These observations are in support of a previous report that the I-antigen activity of erythrocytes can be destroyed by a mixture of galactosidase and β -N-acetylglucosaminidase (Marcus *et al.*, 1963). However, the interpretation of these results is tentative at the present time, for structural changes induced by the enzyme treatments could not be studied because of insufficient material. Sialosyl residues are apparently not involved in any of the Ii-antigen specificities; in fact, additional determinants recognized by antibodies from patients Da and McDon may have been revealed after their removal. The results with anti-(blood-group I) Ma and anti-(blood-group i) McC, Tho and Den indicate that the structures recognized by them are masked by sialosyl residues in the intact gangliosides. The antigenic determinants recognized by the anti-(blood-group I) Da and anti-(blood-group i) McDon were enhanced to the same extent by acid treatment and by neuraminidase. On the other hand, the acid treatment was more effective than neuraminidase in revealing the determinants reactive with anti-(blood-group I) Ma and anti-(blood-group i) McC and Tho. Activity with anti-(blood-group i) Den was only demonstrable in the acid-treated gangliosides. Neuraminidase usually removes sialic acid residues that are linked to the terminal end of oligosaccharide chains; by acid hydrolysis internally substituted sialic acid residues are additionally removed. [In brain gangliosides, the sialosyl residue linked to internal galactose is usually resistant to hydrolysis by *Clostridium perfringens* neuraminidase, but becomes susceptible in the

presence of sodium taurocholate (Wenger & Wardell, 1973) or below the critical micelle concentration (Rauvala, 1976), whereas all sialosyl residues are hydrolysed by weak acid catalysis.] The new or enhanced activities of the desialylated gangliosides could be due to improved accessibility of already partially exposed haptens (the haptens could be improved by unmasking of important additional residues or by acquiring more optimal orientation on the liposomes) or to the revealing of entirely new haptens. The differences in the reactions of the acid-treated and the neuraminidase-treated fractions with anti-(blood-group I) Ma and anti-(blood-group i) McC, Tho and Den suggest that there are at least two types of sialosyl substitutions. These data strongly suggest that the I and i antigens may be the precursors or core structures, not only of blood-group-active glycoproteins in secretions, but of certain carbohydrate chains found on erythrocytes.

If the I(g) fractions are indeed monosialosyl gangliosides, the different sialosyl substitutions must be on different molecules in the I(g)3Ac-I(g)5Ac pool, and, from what is known about the reciprocal expression of I and i determinants on erythrocytes (Race & Sanger, 1962), the revealed determinants reactive with anti-(blood-group I) Ma are likely to be different from those reactive with the anti-(blood-group i) antibodies. Further studies by methylation analysis and mass spectrometry of intact as compared with enzyme-treated (unpooled) I(g)Ac fractions should give complete information on these antigenic determinants. The present study deals predominantly with the inhibitory activities of the I(g) and I(h) fractions in radioimmunoassays. The finding of blood-group-H structure in the gangliosides is also of considerable interest (Suzuki *et al.*, 1975; Wiegandt, 1973). The occurrence of blood-group-A and -B activities on similar gangliosides from donors of A and B types now deserves investigation.

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