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The reactivities of human erythrocyte autoantibodies anti-Pr_2, anti-Gd, Fl and Sa with gangliosides in a chromatogram binding assay

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The thin layer chromatogram binding assay was used to study the reaction of several natural-monoclonal autoantibodies which recognize sialic acid-dependent antigens of human erythrocytes. Immunostaining of gangliosides derived from human and bovine erythrocytes was achieved with four autoantibodies designated anti-Pr_2, anti-Gd, Sa and Fl, each of which has a different haemagglutination pattern with untreated and proteinase-treated erythrocytes and with cells of I and i antigen types. From the chromatogram binding patterns of anti-Pr_2 with gangliosides of the neolacto and the ganglio series, it is deduced that this antibody reacts best with *N*-acetylneuraminic acid when it is α2–3- or α2–6-linked to a terminal Gal(β1-4)Glc/GlcNAc sequence and to a lesser extent when it is α2–3-linked to a terminal Gal(β1-3)GalNAc sequence or to an internal galactose and when it is α2–8-linked to another, internal *N*-acetylneuraminic acid residue. The other three antibodies differ from anti-Pr_2 in their lack of reaction with glycolipids of the ganglio series. They react with the NeuAc(α2-3)Gal(β1-4)Glc/GlcNAc sequence as found in G_{M3} and in glycolipids of the neolacto series, but show a preference for the latter, longer sequences. Thus all four antibodies react with sialylated oligosaccharides containing i type (linear) and I type (branched) neolacto backbones. Fl antibody differs from the other three in its stronger reaction with branched neolacto sequences in accordance with its stronger agglutination of erythrocytes of I rather than i type. The four antibodies show a specificity for *N*-acetyl- rather than *N*-glycolyl-neuraminic acid.

Among the erythrocyte autoantibodies associated with cold agglutinin disease of man, several are known to be monoclonal antibodies directed against sialic acid-containing determinants (Roelcke, 1974). These are distinct from the more commonly occurring monoclonal autoantibodies, anti-I and anti-i cold agglutinins, which do not require sialic acid and are directed against the developmentally regulated, branched and linear oligosaccharides of the poly(*N*-acetyl-lactosamine) (neolacto) series, respectively (Feizi, 1981). Five main types of sialic acid-dependent erythrocyte autoantibodies can be distinguished according to their agglutination reactions with native and proteinase-treated erythrocytes and with cells of I and i antigen types (Roelcke, 1973*a*, 1981*a*,*b*; Roelcke *et al.*, 1976, 1977, 1980). The first type are known as anti-Pr antibodies which agglutinate human erythrocytes irrespective of their Ii antigen content; their agglutination is abolished after proteinase treatment of the erythrocytes. Anti-Pr antibodies can be subdivided into three groups (Pr_{1-3}) on the basis of their differing reactivities with erythrocytes of various animal species and with chemically modified erythrocyte glycoproteins. The second type, termed anti-Gd, differ from anti-Pr in that they agglutinate untreated and proteinase-treated cells to the same extent. The third are antibodies of Sa-type; these show reduced agglutination of proteinase-treated cells. The

Abbreviations used: NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; Gal, galactose; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; Glc, glucose; Cer, ceramide.

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fourth and fifth types are two recently described autoantibodies Fl and Lud which react more strongly with adult (I type) than cord blood (i_{cord}) erythrocytes; the latter reacts well with adult erythrocytes of i type (i_{adult}), while the former resembles anti-I antibodies in its lack of reaction with i_{adult} erythrocytes.

These sialic acid-recognizing antibodies are of considerable biological interest for, like the anti-I and anti-i antibodies (Feizi, 1982), they have been found to detect some marked changes in antigenicity associated with cell differentiation in the mouse (K. Uemura, J. Pennington & T. Feizi, unpublished work). Thus it will be important to know the precise antigenic determinants that they recognize.

In the present studies we have used the technique of immuno-t.l.c. to study the reactivities of eight sialic acid-dependent erythrocyte autoagglutinins with a variety of sialoglycolipids (gangliosides). With four autoantibodies of different types, anti-Pr_2, anti-Gd, Sa and Fl, immunostaining was successfully achieved and an array of antigenically active glycolipids revealed. Evidence is presented that all four antibodies react to varying degrees with G_{M3} and sialylated glycolipids of the neolacto series while anti-Pr_2 reacts in addition with those of the ganglio series.

Experimental

Antibodies

The following antibodies from patients with cold agglutinin disease have been described previously: anti-Pr_{1d} (from patient Rob), anti-Pr_{1h} (patient RK), anti-Pr_2 (patient LTh) and anti-Pr_3, anti-Gd (patient Kn); and antibodies of patients Sa, Fl and Lud (references are given in Table 1). The antibodies Sa and Fl were studied as eluates from human erythrocytes [absorbed at 4°C and eluted at 37°C as described previously (Roelcke, 1981*a*,*b*)] and the others were studied using whole plasma. The information available on the immunoglobulin types, haemagglutination titres and the effect of proteinase treatment of erythrocytes on haemagglutination by these antibodies is summarized in Table 1.

Gangliosides

The following gangliosides (structures shown in Table 2) were purified as described previously; G_{M1} from canine brain (Kunishita *et al.*, 1979); G_{M2} from the brain of a Tay–Sachs disease patient (Kawamura & Taketomi, 1977); G_{D1a}, G_{D1b} and G_{T1b} from bovine brain (Momoi *et al.*, 1976).

Human erythrocyte gangliosides were prepared from pooled outdated blood group B erythrocyte stroma by chromatography of Folch upper phase lipids (Folch *et al.*, 1957) on DEAE-Sephadex A-25 (Momoi *et al.*, 1976). The ganglioside fraction eluted with 0.05–0.1 M-ammonium acetate in methanol was used. Sialosylneolactotetraosylceramide, *N*-acetyl type (Siddiqui & Hakomori, 1973), was purified from the human erythrocyte ganglioside mixture by preparative t.l.c. on a silica gel 60 HP-TLC plate (E. Merck, Darmstadt, Germany). A fraction of bovine erythrocyte gangliosides enriched in sialosylneolactotetraosylceramide, *N*-glycolyl type, and more complex gangliosides were prepared by silicic acid column chromatography (Uemura *et al.*, 1978). Sialosylneolactotetrasylceramide containing both *N*-glyco-

Table 1. *Summary of the information available on the immunoglobulin type and the haemagglutinating properties of the human autoantibodies against N-acetylneuranimic acid-dependent antigens used in this study*

For each autoantibody the titre (reciprocal of the highest dilution giving haemagglutination) is given when normal erythrocytes from adults (I-type) were used. With the exception of Fl and Lud, the antibodies have been shown to react equally well with erythrocytes of i type. Antibody Fl shows negligible agglutination of erythrocytes of i_{cord} and i_{adult} types. Antibody Lud reacts strongly with i_{adult} but not with i_{cord} erythrocytes. In the present study antibodies Sa and Fl were used as eluates from erythrocytes of I-type; the other antibodies were studied in plasma.

Antibodies	Immunoglobulin type	Haemagglutination titre (4°C) against human erythrocytes	Effect of proteinase on haemagglutination*	References
Anti-Pr_2 (LTh)	IgMK	1000	↓	Roelcke (1973*a*); Roelcke *et al.* (1976)
Anti-Gd (Kn)	IgMK	64	–	Roelcke *et al.* (1977)
Sa	IgMK	2000	(↓)	Roelcke *et al.* (1980)
Fl	IgM	256	–	Roelcke (1981*b*)
Lud	IgM	64	(↓)	Roelcke (1981*a*)
Anti-Pr_3	IgMK	512	↓	
Anti-Pr_{1d} (Rob)	IgAK	3200	↓	Roelcke (1973*a*);
Anti-Pr_{1h} (RK)	IgAK	4000	↓	Roelcke *et al.* (1976)

* ↓, haemagglutination markedly reduced, (↓) partially reduced, –, unaffected by proteinase treatment.

Table 2. *Summary of the reactivities of erythrocyte and brain gangliosides with anti-Pr_2 antibody*
The nomenclature of Svennerholm (1963) is used for brain gangliosides. The degree of immunostaining of each ganglioside in the chromatogram binding assay is shown as: −, negative; (+), weak; +, moderate; + +, strong; + + +, very strong. Unless indicated otherwise, the gangliosides were examined using 1 μg of purified samples.

Designation	Carbohydrate sequence	Immunostaining
1. G_{M3}	NeuAc(α2-3)Gal(β1-4)Glc(β1-1)Cer	+ + +
2. Sialosylneolactotetraosylceramide (NeuAc)	NeuAc(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc(β1-1)Cer	+ + +
3. Sialosylneolactohexaosylceramide (NeuAc)	NeuAc(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc(β1-1)Cer	+ + +†
4. G_{Dla}	NeuAc(α2-3)Gal(β1-3)GalNAc(β1-4)Gal(β1-4)Glc(β1-1)Cer | 2,3 NeuAcα	+ +
5. G_{D3}	NeuAc(α2-8)NeuAc(α2-3)Gal(β1-4)Glc(β1-1)Cer	+*
6. G_{D2}	GalNAc(β1-4)Gal(β1-4)Glc(β1-1)Cer | 2,3 NeuAcα | 2,8 NeuAcα	+*
7. G_{Dlb}	Gal(β1-3)GalNAc(β1-4)Gal(β1-4)Glc(β1-1)Cer | 2,3 NeuAcα | 2,8 NeuAcα	+
8. G_{Tlb}	NeuAc(α2-3)Gal(β1-3)GalNAc(β1-4)Gal(β1-4)Glc(β1-1)Cer | 2,3 NeuAcα | 2,8 NeuAcα	+
9. G_{M2}	GalNAc(β1-4)Gal(β1-4)Glc(β1-1)Cer | 2,3 NeuAcα	(+)
10. G_{M1}	Gal(β1-3)GalNAc(β1-4)Gal(β1-4)Glc(β1-1)Cer | 2,3 NeuAcα	(+)
11. G_{M3}(NeuGc)	NeuGc(α2-3)Gal(β1-4)Glc(β1-1)Cer	−
12. Sialosylneolactotetraosylceramide (NeuGc)	NeuGc(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc(β1-1)Cer	−†
13. Sialosylneolactohexaosylceramide (NeuGc)	NeuGc(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc(β1-1)Cer	−†

* Gangliosides stained in human brain ganglioside mixture.
† Gangliosides examined in bovine erythrocyte ganglioside mixture.

lyl type (90%) and *N*-acetyl type (10%) was purified as described previously (Uemura *et al.*, 1978). G_{M3} of *N*-acetyl and *N*-glycolyl types were separated (Uemura *et al.*, 1978) from the bovine erythrocyte G_{M3} mixture by preparative t.l.c. as above. Human brain gangliosides were prepared according to Ledeen *et al.* (1973) from a normal cerebrum. A glycolipid fraction enriched in sialosylneolactohexaosylceramide from human chronic granulocytic leukaemia cells (Macher *et al.*, 1982) was a gift from Dr. Bruce Macher, University of California School of Medicine, Cancer Research Institute, San Francisco, CA, U.S.A.

Glycolipid mixtures from human erythrocytes

Blood group O blood of I-antigen type was collected in anticoagulant/citrate/dextrose (Mollison, 1979) from a healthy adult (designated OI), an adult with i type erythrocytes (i_{adult}) and three neonates, pooled (i_{cord}). The OI and Oi_{cord} erythro-

cytes were used after storage at 4°C for 5 days and the i_{adult} cells at −20°C in glycerol for 9 months according to Mollison (1979). From erythrocyte membranes (Dodge *et al.*, 1963), glycolipids were extracted (Uemura *et al.*, 1983) and evaporated to dryness. The dried residue was treated with 0.1 M-NaOH for 1 h at room temperature and dialysed against water, evaporated and finally dissolved in chloroform/methanol (1:1, v/v).

Immuno-t.l.c.

Glycolipids were chromatographed on HP-TLC plates (aluminium sheets, silica gel 60, E. Merck) using a solvent system of chloroform/methanol/0.5% $CaCl_2 2H_2O$ in water (11:9:2, by vol.). The following antibodies were used: anti-Pr_2 (1:300 dilution in phosphate-buffered saline containing 5% albumin); Gd, Sa, anti-Pr_{1d} and anti-Pr_{1h} (1:100 dilution); Lud and anti-Pr_3 (1:30 dilution); Fl (1:10 dilution). Normal serum supplemented with human immunoglobulin M (10 mg/ml) was used as a control (1:50 dilution). The binding of antibodies to glycolipids on t.l.c. plates was detected by overlaying with the human autoantibodies followed by ^{125}I-labelled rabbit immunoglobulins to human μ or α chains and autoradiography as described previously (Magnani *et al.*, 1981; Uemura *et al.*, 1983).

In a preliminary experiment the effect of calcium ions on immunostaining by anti-Pr_2 antibodies was studied. No difference was found in the intensity of immunostaining when 0.1 mM-$CaCl_2$ or 10 mM-EDTA (tetrasodium salt) was added to the 5% albumin in phosphate-buffered saline used for the pretreatment of the plates and as a diluent of the antibodies.

Results

With four of the eight autoantibodies tested, anti-Pr_2, anti-Gd, Sa and Fl, immunostaining of human and bovine erythrocyte gangliosides was readily demonstrated (Figs. 1A and 1B). With anti-Pr_2 there was in addition immunostaining of human brain gangliosides in the region of G_{M3}, G_{M1}, G_{D1a}, G_{D1b} and G_{T1b} (Fig. 1C). Weak immunostaining was visualized with antibody Lud and bovine gangliosides (results not shown), but with the Pr_3 and two Pr_1 antibodies there was no immunostaining under the conditions used. These four antibodies were not investigated further.

Glycolipids expressing the Pr_2 determinant

The ability of anti-Pr_2 to react with brain gangliosides was investigated further by immunostaining of purified components (Fig. 2). This

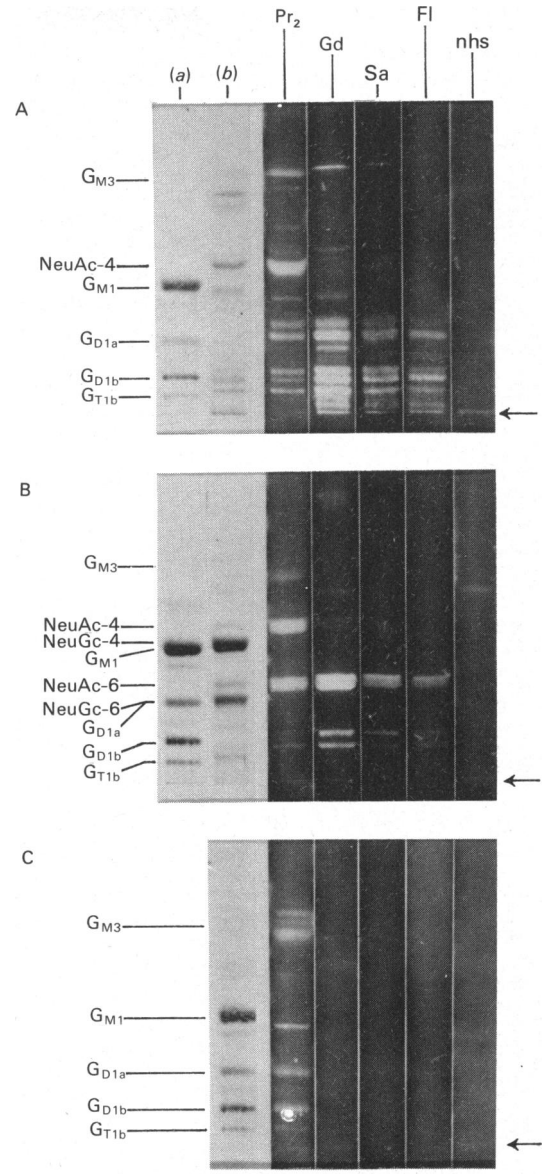


Fig. 1. *Reactions of the autoantibodies anti-Pr_2, anti-Gd, Sa and Fl with human erythrocyte gangliosides from blood group B donors (panel A), bovine erythrocyte gangliosides (panel B), and human brain gangliosides (panel C)*

Each ganglioside preparation, containing 1.7 μg, 1.5 μg and 1.9 μg of sialic acid, respectively, was chromatographed on HP-TLC plates and immunostained with the autoantibodies as described in the Experimental section. nhs indicates normal human serum supplemented with human immunoglobulin M (10 mg/ml) as a control. Lane (*a*) shows orcinol staining of human brain gangliosides as reference, and lane (*b*), orcinol staining of the glycolipids used for immunostaining in each plate. The positions of the major brain gangliosides G_{M1}, G_{D1a}, G_{D1b}, G_{T1b}, the *N*-acetyl and *N*-glycolyl isomers of sialosylneolactotetraosylceramide (abbreviated to NeuAc-4 and NeuGc-4) and of sialosylneolacto-

hexaosylceramide (abbreviated to NeuAc-6 and NeuGc-6) are indicated. Arrows indicate points of application.

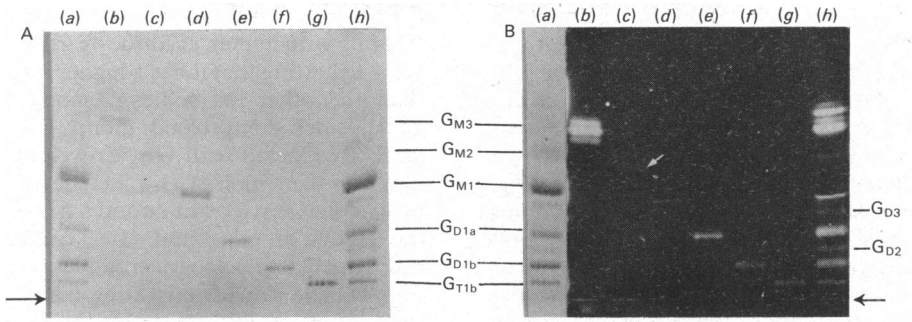


Fig. 2. *Reactions of anti-Pr_2 autoantibody with purified brain gangliosides and a mixture of total brain gangliosides detected by immunostaining on t.l.c. plates*

Plate A and lane (*a*) in plate B were visualised with orcinol reagent. Plate B, lanes (*b–h*) show the autoradiogram after immunostaining with anti-Pr_2 as described in the Experimental section. Lane (*a*) contains human brain ganglioside mixture (1.9 μg of sialic acid); (*b*), G_{M3}; (*c*), G_{M2} containing a trace amount of G_{D1a}; (*d*), G_{M1}; (*e*), G_{D1a}; (*f*), G_{D1b}; (*g*), G_{T1b} (approx. 1 μg of glycolipid each); (*h*), human brain gangliosides (1.9 μg of sialic acid). G_{D3} and G_{D2} in the ganglioside mixture gave a positive reaction. White arrow indicates weak staining of G_{M2}.

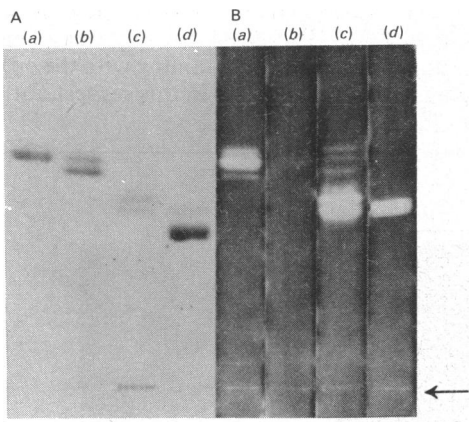


Fig. 3. *Reactions of anti-Pr_2 autoantibody with gangliosides containing N-acetylneuraminic acid but not with those containing N-glycolylneuraminic acid shown by immunostaining on t.l.c. plates*

Plate A was visualized with orcinol reagent and plate B shows the autoradiogram after immunostaining with anti-Pr_2 as described in the Experimental section. Lane (*a*) contains the NeuAc isomer of G_{M3} (structure 1), 1 μg; (*b*), NeuGc isomer of G_{M3} (structure 11), 1 μg; (*c*), NeuAc form of sialosylneolactotetraosylcermamide (structure 2), 0.5 μg; (*d*), NeuGc isomer of sialosylneolactotetraosylceramide (structure 12), 1 μg from bovine erythrocytes, consisting of 90% structure 12 (the lower band in panel A) and 10% structure 2 (the upper band). Double bands in plate A, lanes (*a*), (*b*) and (*c*) are due to differences in the ceramide portion of the gangliosides. Note the presence of a trace amount of G_{M3} in the sialosylneolactotetraosylceramide preparation detected by immunostaining (plate B, lane *c*) and the strong staining of the contaminating NeuAc isomer (structure 2) of the preparation of NeuGc neolactotetraosylceramide (structure 12) in plate B, lane (*d*).

antibody reacted very strongly with G_{M3} (structure 1, Table 2), strongly with G_{D1a} (structure 4), moderately strongly with G_{D1b} and G_{T1b} (structures 7 and 8) and weakly with preparations of G_{M2} and G_{M1} (structures 9 and 10). These observations, together with the moderately strong reactivity of G_{D3} and G_{D2} (structures 5 and 6) in the human brain ganglioside mixture (Fig. 2B) and the strong reaction of sialosylneolactotetraosylceramides (structure 2) of human and bovine erythrocytes (Figs. 1A and 1B) and sialosylneolactohexaosylceramide (structure 3) of bovine erythrocytes (Fig. 1B) indicated that among the compounds tested the most optimal structure for reaction with this antibody is sialic acid joined by α2–3 linkage to a terminal Gal(β1-4)Glc/GlcNAc sequence. As summarized in Table 2 this antibody reacts less strongly with sialic acid α2–3-linked to a terminal Gal(β1-3)GalNAc sequence or to an internal galactose, as well as sialic acid α2–8-linked to another, internal sialic acid.

The immunostaining in the region of the *N*-acetyl rather than the *N*-glycolyl form of sialosylneolactotetraosylceramide and sialosylneolactohexaosylceramide of bovine erythrocyte gangliosides (Fig. 1B) suggested that this antibody reacts with the *N*-acetyl analogues only. This was confirmed using preparations of G_{M3} and sialosylneolactotetraosylceramide enriched for the *N*-acetyl or the *N*-glycolyl forms (Fig. 3). In each case there was immunostaining of the *N*-acetyl forms only.

Glycolipids expressing the Gd, Sa and Fl determinants

The determinants recognized by autoantibodies anti-Gd, Sa and Fl were lacking among brain

gangliosides (Fig. 1C). But they were expressed among gangliosides of human and bovine erythrocytes (Figs. 1A and 1B) which are known to be predominantly of the neolacto-series (Chien *et al.*, 1978; Hakomori, 1981). The majority of immunoreactive glycolipid bands were clustered as two groups, the first migrating in the region of the G_{D1a} standard and sialosylneolactohexaosylceramides and the second in the region of G_{D1b} and G_{T1b} and slow migrating components corresponding to more complex gangliosides of the neolacto series (Feizi *et al.*, 1978; Uemura *et al.*, 1983). Some reactivity in the region of G_{M3} was also present (Fig. 1A and Fig. 4). Several of the bands immunostained by these antibodies had identical mobilities to those reacting with anti-Pr_2. However the relative intensities of immunostaining of these bands differed with the four antibodies as discussed below.

Anti-Gd, Sa and Fl resembled anti-Pr_2 in their positive immunostaining of the *N*-acetyl form of sialosylneolactohexaosylceramide (structure 3) but not the *N*-glycolyl form (structure 13) of bovine erythrocytes (Fig. 1B).

Anti-Gd. Despite its lower haemagglutinating titre (Table 1) anti-Gd gave the strongest immunostaining with human erythrocyte gangliosides (Fig. 1A), suggesting that it has a higher binding affinity than the other antibodies. Among the pool of gangliosides from blood group BI donors this antibody reacted with G_{M3} as well as a triplet of bands in the region of G_{D1a} standard and sialosylneolactohexaosylceramide and a triplet of bands in the region of G_{D1b} and G_{T1b} corresponding to sialosylneolacto-octaosylceramides (Fig. 1A and Fig. 4). The slowest migrating component in the sialosylneolactohexaosylceramide region did not give immunostaining with anti-Pr_2, Sa and Fl. This Gd-active component was not detected among the Folch upper phase glycolipids isolated from a single group OI donor (Fig. 4). Moreover the relative intensities of the other bands in the group OI extract differed from those in the pooled group BI gangliosides.

Anti-Gd differed from anti-Pr_2 in its stronger reaction with sialosylneolactohexaosylceramide of bovine erythrocytes than sialosylneolactotetraosylceramide (Fig. 1B). These two glycolipid bands gave approximately equal staining with the orcinol reagent and with anti-Pr_2. In this respect anti-Gd

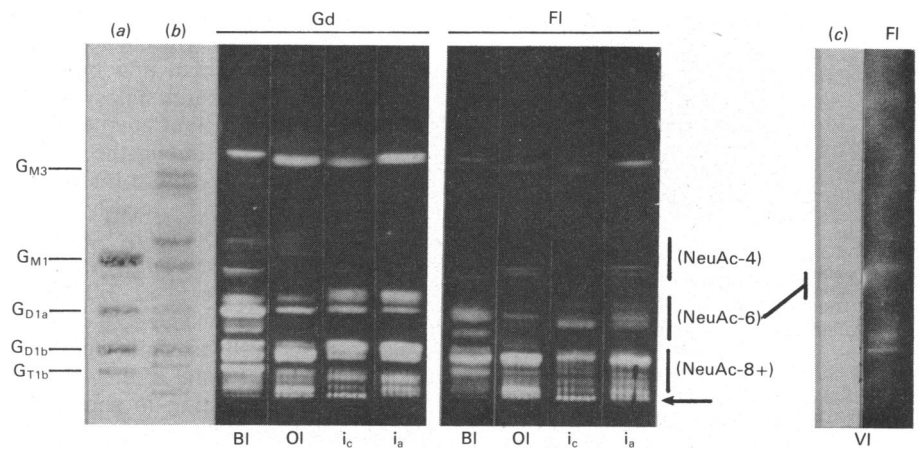


Fig. 4. *Reactions of anti-Gd and Fl autoantibodies with glycolipids of human erythrocytes of I and i type and with a glycolipid fraction enriched in sialosylneolactohexaosylceramide from human, chronic granulocytic leukaemia cells*

Human erythrocyte gangliosides from pooled type B blood (BI) containing 1.7 μg of sialic acid, Folch upper phase glycolipids from 200 μl each of packed type O adult erythrocytes (OI) from a single donor, type Oi_{adult} erythrocytes (i_a) from a single donor and type O_{cord} erythrocytes (i_c), pooled from three individuals and a glycolipid fraction from human chronic granulocytic leukaemia cells enriched in sialosylneolactohexaosylceramide (VI) were chromatographed on HP-TLC plates as described in the Experimental section. Autoradiograms show immunostaining pattern with anti-Gd and Fl. Lane (*a*) shows orcinol staining of human brain gangliosides as a reference, lane (*b*), that of human erythrocyte gangliosides BI and lane (*c*) that of the chronic granulocytic leukaemia gangliosides. Immunostained band clusters migrating in the regions of sialosylneolactotetraosyl (NeuAc-4)-hexaosyl (NeuAc-6) and -octaosyl and larger (NeuAc-8+) ceramides are indicated. Apart from NeuAc-6, fraction VI contains two slow-migrating minor components which were not detectable by orcinol stain but gave immunostaining with Fl antibody. The erythrocyte glycolipids and fraction VI were chromatographed in different experiments; this accounts for their differing migration.

resembles Sa and Fl which also gave weak or inconsistent immunostaining with the short chain structures.

Antibody Sa. The immunostaining pattern with this antibody was similar to that of anti-Gd. The main difference was the lack of reaction with the slow migrating component in the sialosylneolactohexaosylceramide region of the group BI ganglioside pool.

Antibody Fl. Because this antibody resembles anti-I antibodies in its preferential agglutination of erythrocytes of I type rather than those of i_{cord} or i_{adult} types, it was of interest to look for evidence for a preferential binding to branched, sialylated glycolipids of the neolacto series which are abundant in I-type cells and to compare the immunostaining of glycolipids derived from group OI, group Oi_{cord} and Oi_{adult} erythrocytes. Clearly, this antibody gave immunostaining in the region of the linear structures in this series, i.e. in the region of sialosylneolactohexaosylceramides of human (Fig. 1A, Fig. 4) and bovine (Fig. 1B) gangliosides [described by Niemann *et al.* (1978)] and some weak and inconsistent staining in the regions of G_{M3} and sialosylneolactotetraosylceramides. However the band most strongly stained in the gangliosides of pooled group BI erythrocytes and the Folch-upper phase glycolipids of group OI erythrocytes was in the region of GD_{1b} standard corresponding to sialosylneolacto-octaosylceramides.

In addition, antibody Fl gave substantial immunostaining of the Folch upper phase glycolipids from group Oi_{cord} and group Oi_{adult} erythrocytes (Fig. 4). Although the total radioactive counts bound to the chromatograms (18700c.p.m. with OI erythrocytes; 15200c.p.m. with Oi_{cord}; 17600c.p.m. with Oi_{adults}) and the total areas of densitometric scans of the autoradiographs (results not shown) were similar in cells of I and i type, the immunostaining patterns were different in the two cell types. For example, in cells of i type the staining in the sialosylneolacto-octaosylceramide region was relatively less while that in the sialosylneolactohexaosylceramide region was more pronounced than with cells of I type. From immunostaining experiments using anti-I and anti-i antibodies we have recently obtained evidence that the sialosylneolacto-octosylceramides are branched in cells of I type and linear in those of i type (Uemura *et al.*, 1983). The ability of antibody Fl to react with linear structures was confirmed by immunostaining of sialosylneolactohexaosylceramide isolated from chronic granulocytic leukaemia cells (Fig. 4). Thus antibody Fl can react with both the linear and branched structures but has a preference for the branched structures. An incidental observation was the lack of immunostaining in the neolactotetraosylceramide region in i_{cord} erythrocytes.

Discussion

These studies show the successful application of immuno-t.l.c. to reveal a family of *N*-acetylneuraminic acid containing gangliosides recognized by the monoclonal autoantibodies anti-Pr_2, anti-Gd, Sa and Fl.

Anti-Pr_2 was studied in the greatest detail. Because this antibody does not agglutinate papain-treated erythrocytes (Roelcke, 1974, 1981*a*) it has been presumed that the antigenic determinant it recognizes is associated exclusively with the erythrocyte sialoglycoproteins. However, the present studies have clearly shown that this antibody reacts well with sialylated glycolipids (gangliosides) on thin layer chromatograms. Further investigations are required with glycolipid extracts from papain-treated erythrocytes to evaluate their glycolipids and to establish the relative roles of the glycoproteins and glycolipids in the haemagglutination reaction. It is possible that both the sialoglycoproteins and the gangliosides are required in a co-operative manner for haemagglutination to occur and the glycolipid-associated determinants remaining after removal of the glycopeptides may be too sparse for haemagglutination by this antibody. It is of interest that the Pr_2 antigen of dog erythrocytes is unaffected by proteinase treatment (Roelcke, 1973*b*).

Pr_2 antibody gave immunostaining not only of erythrocyte gangliosides but of brain gangliosides also. This is in accord with the immunocytochemical studies of Römer *et al.* (1979), which have shown that this antibody reacts with brain tissues of several mammalian species.

From the immunostaining reactions of anti-Pr_2 with the several gangliosides of the neolacto and the ganglio series and G_{M3} (Table 2) we deduce that the preferred sequence is NeuAc(α2-3)-Gal(β1-4)GlcNAc/Glc, although this antibody may react with any ganglioside having the sequence NeuAc(α2-3)Gal or NeuAc(α2-8)-NeuAc(α2-3)Gal. Since these studies were completed we (P. Scudder & T. Feizi, unpublished work) have shown that anti-Pr_2, but not anti-Gd, gives strong immunostaining of the NeuAc(α2-6) isomer of sialosylparagloboside with the sequence

NeuAc(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc-Cer

isolated from human erythrocytes by Watanabe *et al.* (1979).

Our studies provide an explanation for previous inhibition data with this antibody. These have shown that haemagglutination of erythrocytes by anti-Pr_2 can be inhibited by high concentrations of the α2–3 and α2–6 isomers of sialyl-lactose [NeuAc(α2-3/6)Gal(β1-4)Glc] (Tsai *et al.*, 1977; Roelcke *et al.*, 1980) as well as the oligosaccharides of sialoglycoproteins containing the sequence NeuAc(α2-3)Gal(β1-3)GalNAc (Ebert *et al.*, 1979) and the glycolipids G_{M3} and sialosylparagloboside (Tsai *et al.*, 1977).

In contrast to anti-Pr_2, the antibodies Gd, Sa and Fl do not react with sialoglycolipids of the ganglio series but they react with the NeuAc(α2-3)-Gal(β1-4)Glc/GlcNAc sequences as found in the glycolipids of the neolacto series and G_{M3}. Their immunostaining patterns indicate that they have a considerable preference for long chain structures with backbones of neolactohexaosyl- and longer ceramides.

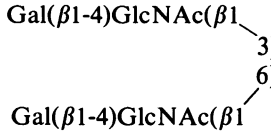
Our observations that anti-Gd reacts with gangliosides in the region of the sialosylneolactohexaosylceramides (structure 3) of human and bovine erythrocytes (Figs. 1A and 1B) support those of Kundu *et al.* (1982) that a purified glycolipid proposed as structure 3 inhibits the agglutination of erythrocytes by this antibody. However, the present studies show that this antibody reacts with G_{M3} and numerous other erythrocyte gangliosides. A high proportion of these are slow migrating components located in the regions of G_{D1b} and more complex structures. Such slow migrating structures were abundant in erythrocytes of I_{adult}, i_{cord} and i_{adult} types with somewhat differing immunostaining patterns in the three cell types (Figs. 1A and 1B; Fig. 4). From t.l.c.-immunostaining experiments (Uemura *et al.*, 1983), using anti-I and anti-i antibodies with specificities for branched or linear backbones of the neolacto series, we have obtained evidence that among these slow migrating gangliosides there are sialylated ceramide octasaccharides with predominantly linear backbones in cells of i-type (as in structure 14, shown below) and branched backbones in those of I-type (as in structure 15); in addition there are more complex structures behaving as sialoglycolipids with decasaccharide and larger backbones in which both linear and branched sequences are present in all three cell types.

The intense reaction of anti-Gd with glycolipids having linear or branched backbones undoubtedly accounts for its lack of discrimination between cells of I and i types.

The majority of the gangliosides of human and bovine erythrocytes that gave immunostaining with antibodies Sa and Fl had mobilities identical to those of the components reacting with anti-Gd. Five of the bands in human erythrocytes and three in bovine erythrocytes also corresponded to those immunostained by anti-Pr_2. A notable difference was the ganglioside in the pooled human erythrocytes of BI type migrating in the sialosylneolactohexaosylceramide region (between G_{D1a} and G_{D1b}) which reacted with anti-Gd but not with the other three antibodies (Figs. 1A and 4). This Gd-reactive component may correspond to the glycolipid isolated by Kundu *et al.* (1982) which inhibited haemagglutination by anti-Gd but not Sa. It will be of interest to perform structural studies of the three immunoreactive glycolipids in this sialosyl neolactohexaosylceramide region.

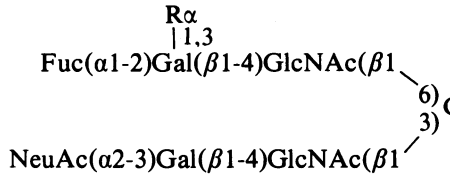
The preferential haemagglutination of I- rather than i-type erythrocytes by Fl antibody (Roelcke, 1981*b*; Table 1) suggested that this antibody might be directed against branched, sialylated oligosaccharides of the neolacto series (sialylated I antigen). However, on thin layer chromatograms this antibody was found to immunostain gangliosides of cells of I and i-type which are known to be predominantly branched in the former cells and linear in the latter (Fig. 1A and Fig. 4). Furthermore this antibody reacts with linear sialosylneolactohexaosylceramide of chronic granulocytic leukemia cells. Two observations indicated a preferential reaction with branched structures: (*a*) although the total counts bound by extracts from I_{adult}, i_{cord} and i_{adult} erythrocytes were comparable, the I_{adult} cells gave more pronounced staining in the 'sialosyl-I' region of group OI erythrocytes; (*b*) although this antibody gave immunostaining in the region of the major 'sialosyl-i' component (structure 3) of bovine erythrocytes (Fig. 1B), the reaction was weak in comparison with that seen with anti-Pr_2, Gd and Sa. Thus, Fl antibody resembles certain anti-I antibodies, such as Step, Ver and Phi, which show preferential reactions with branched neolacto sequences but can react with linear glycolipid structures (Watanabe *et al.*,

Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc-Cer (14)



(15)

1979; Feizi *et al.*, 1979; K. Uemura, P. Hanfland, R. Childs & T. Feizi, unpublished work). After these studies were completed we were informed of the work of Kannagi *et al.* (1983) showing that fucosylated and branched sialoglycolipids with blood group A, H and B activities [as in structure 16 shown below (where R = Gal or GalNAc)] isolated from human erythrocytes react strongly with Fl antibody.



(16)

Our observations are in accord with that finding. However, a requirement for the fucose residue was assumed by Kannagi *et al.* (1983), largely due to the failure of this antibody to react with the corresponding afucosyl ganglioside analogue derived from bovine erythrocytes. This observation must be interpreted with caution since the sialic acid associated with bovine erythrocytes is predominantly *N*-glycolylneuraminic acid (Uemura *et al.*, 1978) which is not recognized by this antibody. This alone would account for a lack of reaction. Kannagi *et al.* (1983) did not specifically isolate the *N*-acetyl from the *N*-glycolylneuraminic acid form of this afucosyl analogue from bovine erythrocytes, although they estimated that it consisted predominantly of the *N*-acetylneuramic acid form (S. Hakomori, personal communication). Moreover, the enzymic removal of fucose residues from structure 16 may not be straightforward and it would be necessary to characterize structurally the afucosyl analogue thus obtained in order to establish the requirement for the fucose residue.

The differences in the relative intensities in immunostaining of the several bands by the four antibodies, suggest that each antibody reacts to a different extent with each component. However, it is conceivable that the glycolipid bands with identical mobilities in human erythrocytes immunostaining with the four antibodies (Fig. 1A) consist of isomers with different structures. Structural studies of the purified components will be required to investigate this possibility. The differences in the fine specificities of these four autoantibodies would also account for the differences in their haemagglutination reactions with various types of native and proteinase-treated erythrocytes. Only certain of the glycolipids may be accessible to each antibody on the intact cell membranes, as suggested by Hakomori & Young (1978).

The differences in the immunostaining of the glycolipids obtained from pooled (outdated) blood group B blood versus those isolated from a fresh sample from a single group O donor deserve some comment. Apart from the blood group and other isotype differences, the possibility of degradative changes and inevitable contamination with leucocytes occurring in the outdated blood should be considered, for the buffy coat is readily separated from erythrocytes in the fresh state, but not from stored outdated blood.

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