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Multiplicity of Molecules carrying Blood-Group-I Antigen on Erythrocyte Membranes

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A human serum containing a monoclonal anti-(blood-group I) antibody was used to investigate the distribution of blood-group-I antigen on erythrocyte membrane components. Sodium dodecyl sulphate/polyacrylamide-gel-electrophoresis profiles of immune precipitates by using ^{3}H-labelled (by the galactose oxidase/NaB^3H_4 method) and ^{125}I-labelled solubilized stroma were compared. Different radioactive profiles were revealed by the two radiolabelling methods. In the immunoprecipitates the predominant ^{125}I radioactivity within the gel had the electrophoretic mobility of Band-3 protein (apparent mol.wt. 90000–100000), whereas the ^{3}H radioactivity revealed a diffusely migrating component(s) (apparent mol.wt. range 40000–70000) in addition to radioactivity compatible with glycolipids at the dye front. The diffusely migrating ^{3}H-labelled component was shown to have a similar electrophoretic mobility to a subpopulation of erythrocyte poly(glycosyl)ceramides with blood-group-I activity.

The blood-group-I and -i antigens are carbohydrate structures on the surface of adult and cord blood erythrocytes respectively and are defined by human autoantibodies, many of which are monoclonal immunoglobulin M proteins (Feizi, 1977). It is now established that the majority of blood-group-i antigenic determinants are expressed on the straight-chain carbohydrate sequence:

Galβ1→4GlcNAcβ1→
3Galβ1→4GlcNAcβ1→3Gal—

(Niemann *et al.*, 1978) and the majority of blood-group-I determinants on the branched structure

Galβ1→4GlcNAcβ1
↘
$^6_3$Galβ1→4GlcNAcβ1→3Gal—
↗
Galβ1→4GlcNAcβ1

(Watanabe *et al.*, 1979; Feizi *et al.*, 1979). Thus the monoclonal anti-(blood-group I and i) autoantibodies are powerful reagents for detecting and isolating macromolecules carrying these two types of carbohydrate antigen. A recent affinity-chromatographic study of radioiodinated solubilized erythrocyte membranes, by using a monoclonal anti-(blood-group I) antibody column as an immunoadsorbent, revealed blood-group-I activity associated with Band-3 protein (Childs *et al.*, 1978). Earlier immunoprecipitation studies (Feizi, 1978) with ^{3}H-labelled erythrocyte membranes (by the galactose oxidase/NaB^3H_4 method) had revealed blood-group-I activity on an unidentified component(s) migrating diffusely in SDS/polyacrylamide gels in the apparent mol.wt. range 40000–70000; however, there was negligible radioactivity in the region of Band-3 protein (approximate mol.wt. 100000). In the present studies we have investigated these different observations by immunoprecipitation studies by using the same erythrocyte membranes radiolabelled with both ^{3}H and ^{125}I. We have confirmed that different blood-group-I-active components are revealed by the two labels. Comparative studies with ^{3}H-labelled erythrocyte poly(glycosyl)ceramide fractions indicate that the ^{3}H-labelled diffusely migrating erythrocyte component(s) may contain a subpopulation of poly(glycosyl)ceramides.

Abbreviation used: SDS, sodium dodecyl sulphate.

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Materials and Methods

SDS was purchased from B.D.H. (Poole, Dorset, U.K.), acrylamide from Bio-Rad (Richmond, CA, U.S.A.), *NN'*-methylenebisacrylamide from Eastman Kodak Co. (Rochester, NY, U.S.A.), NCS solubilizer from Amersham/Searle (Arlington Heights, IL, U.S.A.), and Empigen BB from Albright and Wilson (Whitehaven, Cumbria, U.K.); NaB^3H_4 (10Ci/mmol) and carrier-free ^{125}I were from The Radiochemical Centre (Amersham, Bucks., U.K.); D-galactose oxidase (EC 1.1.3.9; specific activity 714units/mg of protein) was from Kabi Vitrum

(Stockholm, Sweden). All other reagents were purchased from B.D.H. and of AnalaR quality.

Serum containing a high-titre monoclonal anti-(blood-group I) antibody was from patient Step and has been described previously (Feizi & Kabat, 1972; Watanabe *et al.*, 1979; Feizi *et al.*, 1979).

Human monoclonal immunoglobulin M without known antibody specificity was isolated from the serum of patient Ham with Waldenström macroglobulinaemia by Pevikon-block electrophoresis (Müller-Eberhard & Kunkel, 1956). An antiserum was raised against this protein in rabbits and was used after absorption with washed blood-group-A, -B and -O erythrocytes.

A blood-group-I-active poly(glycosyl)ceramide preparation (subfraction 9A) (Kościelak *et al.*, 1976), isolated from human erythrocytes, was a gift from Dr. J. Kościelak (Institute of Haematology, Warsaw, Poland).

Radiolabelling and solubilization of erythrocyte membranes

Blood from a group-O donor was stored in anticoagulant citrate dextrose at 4°C for up to 24h. Erythrocytes were labelled externally with ^3H by the galactose oxidase/NaB^3H_4 method (Gahmberg, 1976) and stroma was prepared from the ^3H-labelled erythrocytes by the method of Dodge *et al.* (1963). The stroma was suspended in 50mM-Tris/HCl, pH8.2, at a protein concentration of 1mg/ml [determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard] and solubilized by adding Empigen BB to a final concentration of 1% (v/v) at 0°C for 60min. Insoluble material was removed by centrifugation at 100000*g* at 2°C for 60min. The ^3H-labelled supernatant was either used directly or after additional labelling with ^{125}I by the chloramine-T method as described previously (Childs *et al.*, 1978).

^3H radioactivity was measured in a Packard Tri-Carb scintillation counter by using 6ml of scintillation fluid with the following composition: 1 litre of toluene containing 7.5g of 2,5-diphenyloxazole and 0.15g of 1,4-bis-(5-phenyloxazol-2-yl)-benzene plus 500ml of Triton X-100. The specific radioactivity of the ^3H-labelled supernatant was 1.5×10^7c.p.m./mg of protein. The ^{125}I γ-radioactivity of the $^3H/^{125}I$-labelled supernatant as measured in a Nuclear Enterprises 1600 gamma counter was 1×10^8c.p.m./mg of protein.

Preparation and radiolabelling of poly(glycosyl)-ceramide-rich fraction from erythrocyte membranes

Crude glycoprotein fraction was prepared from erythrocyte membranes and a poly(glycosyl)ceramide-rich fraction isolated by chromatography on a DEAE-cellulose column as described by Dejter-Juszynski *et al.* (1978). This fraction (equivalent to 1ml of packed stroma), in 400μl of 50mM-sodium phosphate buffer, pH7.4, was treated with 5 units of galactose oxidase at room temperature for 18h and then labelled with ^3H by the addition of 1mCi of NaB^3H_4 (10μl of a 100mCi/ml solution in 1M-NaOH). After 4h at room temperature 1mg of $NaBH_4$ was added and the labelled poly(glycosyl)-ceramides were recovered by gel filtration on a Sephadex G-25 column (20cm$\times$0.7cm) equilibrated in 1% (v/v) Empigen in 50mM-Tris/HCl, pH8.2. The total radioactivity in this fraction was 6.5×10^6 c.p.m.

Radiolabelling of blood-group-I-active poly(glycosyl)-ceramide sub-fraction 9A

A sample (12μg) of this fraction in 25μl of 50mM-sodium phosphate buffer, pH7.4, was labelled with ^3H by the galactose oxidase/NaB^3H_4 method as described above. The specific radioactivity was 3×10^5c.p.m./μg.

Immunoprecipitation

Immunoprecipitates were obtained by a double-antibody method: 20μl of a 1:10 dilution of the human anti-(blood-group I) serum (or normal human serum as a control) was added to the following radiolabelled materials: (*a*) 400μl of ^3H-labelled solubilized stroma containing 1.9×10^6c.p.m.; (*b*) 500μl of $^3H/^{125}I$-labelled solubilized stroma containing 2.1×10^7c.p.m. of ^{125}I; (*c*) 500μl of ^3H-labelled erythrocyte poly(glycosyl)ceramide-rich fraction containing 6.5×10^6c.p.m.; (*d*) 230μl of ^3H-labelled poly(glycosyl)ceramide sub-fraction 9A containing 3.9×10^5c.p.m. After incubation at 0°C for 2h, 4μg of human immunoglobulin M was added as carrier, followed by an excess (100μl) of rabbit anti-(human immunoglobulin M); after further incubation for 2h, precipitates were obtained by centrifugation at 2000*g* for 20min through 1ml of a solution containing 5% (w/v) sucrose and 1% (v/v) Empigen in 50mM-Tris/HCl, pH8.2, and washed with 3$\times$1ml of a solution containing 0.5% (v/v) Empigen in 50mM-Tris/HCl, pH8.2. The precipitates were solubilized by heating at 100°C for 5min in 100μl of a solution containing 5% (w/v) SDS, 2M-urea, 2.5% (v/v) 2-mercaptoethanol and 5% (w/v) sucrose in 10mM-Tris/HCl, pH6.8. Samples (10μl) of the solubilized precipitates were removed for measurement of radioactivity, and the remainder was analysed by SDS/polyacrylamide-gel electrophoresis after the addition of Bromophenol Blue at a final concentration of 0.05mg/ml.

It was important to investigate whether the double-antibody immunoprecipitation method used was effective in precipitating blood-group-I-active macromolecules, and to what extent 1% (v/v)

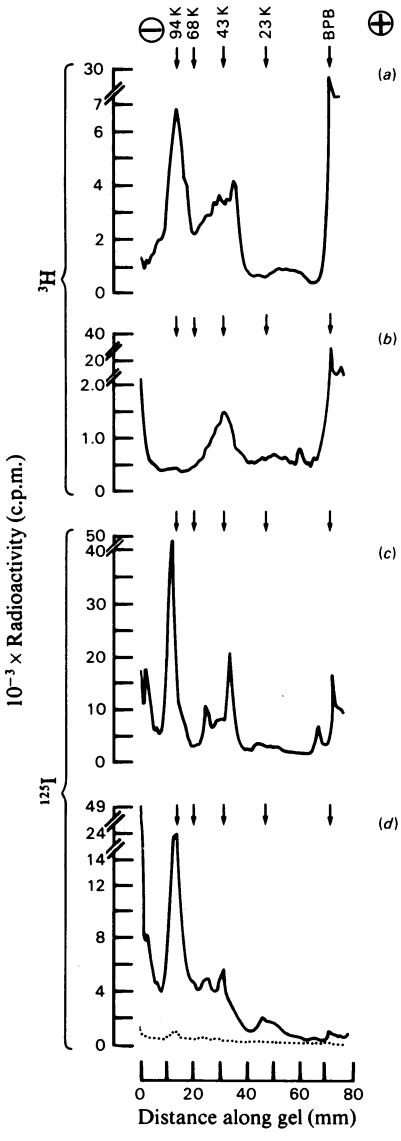

Fig. 1. *SDS/polyacrylamide-gel-electrophoresis profiles of radiolabelled erythrocyte membranes and their anti-(blood-group I) immune precipitates*

Electrophoresis was performed as described in the text; migration was from left to right. (*a*) ^{3}H-labelled solubilized erythrocyte membranes containing 3.5×10^5c.p.m.; (*b*) anti-(blood-group I) immunoprecipitate obtained with the ^{3}H-labelled solubilized membranes containing 1.9×10^5c.p.m.; (*c*) ^{3}H/^{125}I-labelled solubilized erythrocyte membranes containing 4.7×10^6c.p.m. of ^{125}I; (*d*) immunoprecipitates obtained with anti-(blood-group I) serum (solid line) and normal human serum (broken line) by using ^{3}H/^{125}I-labelled solubilized membranes, containing 4.1×10^5 and 2.1×10^4c.p.m. of ^{125}I, respectively. Molecular-weight markers (indicated by arrows) were: phosphorylase *a* (94000); bovine serum albumin (68000); ovalbumin (43000); trypsin (23000); Bromophenol Blue (BPB).

Empigen affected the precipitation. As a reference antigen, a radioiodinated blood-group-I-active glycoprotein isolated from sheep gastric mucosa was used which had been specifically enriched for blood-group-I activity by affinity chromatography on an anti-(blood-group I) antibody adsorbent (Wood *et al.*, 1979). The following precipitation experiments were carried out:

(*a*) double-antibody precipitation as described above, by using 1.4×10^4c.p.m. of labelled sheep glycoprotein in 500μl of 50mM-Tris/HCl buffer, pH8.2;

(*b*) double-antibody precipitation as in (*a*), but by using 1% (v/v) Empigen in the Tris/HCl buffer;

(*c*) co-precipitation of the labelled antigen (1.4×10^4c.p.m. in 50mM-Tris/HCl buffer, pH8.2) in the presence of 10μg of unlabelled blood-group-I-active sheep glycoprotein, and 20μl of a 1:10 dilution of the anti-(blood-group I) serum [in the absence of immunoglobulin M carrier and rabbit anti-(human immunoglobulin)].

At the time of these experiments 86% of the radioactivity in the labelled antigen was precipitable by ethanol at a final concentration of 90% (v/v). The radioactivity precipitated in Expts. (*a*)–(*c*) was 1.2×10^4 (86%), 1.0×10^4 (71%) and 1.1×10^4 (79%) c.p.m. respectively, indicating that the double-antibody method was an effective precipitation method and it was not substantially affected by the detergent used.

Polyacrylamide-gel electrophoresis

Electrophoresis was performed at 100V in 7.5% polyacrylamide (acrylamide/*NN'*-methylenebisacrylamide, 37:1) disc gels containing 0.1% SDS and 0.5M-urea by the system of Laemmli (1970). The gels were frozen at −70°C and cut into 1mm slices with a Mickle gel slicer (Mickle Laboratory Engineering Co., Gomshall, Surrey, U.K.). Gel slices were counted directly for ^{125}I γ-radioactivity. For counting ^{3}H radioactivity the gel slices were first treated with 0.6ml of aq. 90% (v/v) NCS solubilizer at 50°C for 2h and then mixed with 6ml of scintillation fluid.

Results

Polyacrylamide-gel-electrophoresis patterns of radiolabelled solubilized erythrocyte membranes and the immunoprecipitates obtained with the anti-(blood-group-I) serum

The radioactivity profile of the ^{3}H-labelled solubilized stroma (Fig. 1*a*) was similar to that described by Gahmberg (1976). The greatest radio-

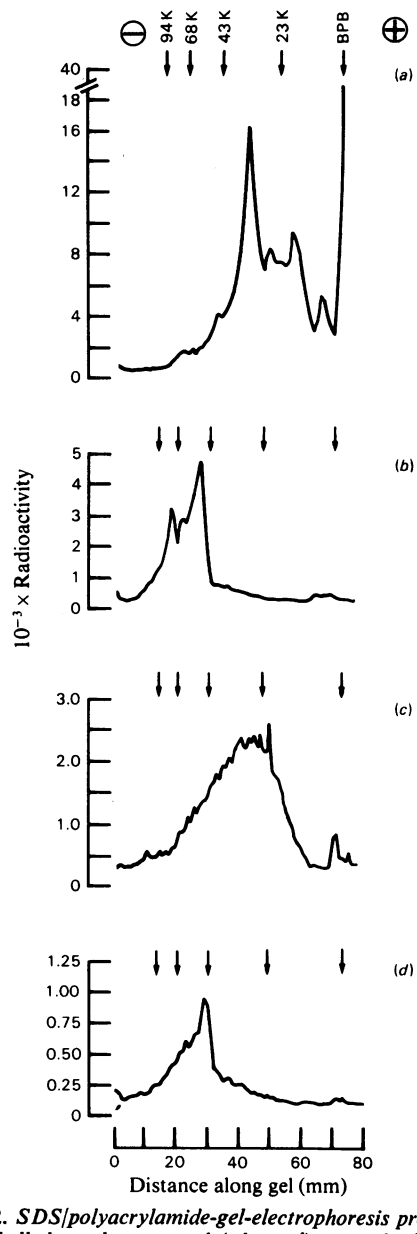

Fig. 2. *SDS/polyacrylamide-gel-electrophoresis profiles of ^{3}H-labelled erythrocyte poly(glycosyl)ceramide fractions and their anti-(blood-group I) immunoprecipitates*

Electrophoresis was performed as described in the text; migration was from left to right. (*a*) ^{3}H-labelled poly(glycosyl)ceramide-rich fraction (prepared as described by Dejter-Juszynski *et al.*, 1978) containing 6×10^5 c.p.m.; (*b*) anti-(blood-group I) immunoprecipitate of the above fraction containing 6.6×10^4 c.p.m.; (*c*) ^{3}H-labelled poly(glycosyl)ceramide subfractions 9A containing 1×10^5 c.p.m.; (*d*) anti-(blood-group I) immunoprecipitate of sub-fraction 9A containing 1.1×10^4 c.p.m. Molecular-weight markers were as described in Fig. 1.

activity (approx. 70% of that applied) was at the dye front coincident with the migration of low-molecular-weight glycolipids. Within the gel there were three main areas of radioactivity in the apparent mol.wt. ranges 70000–110000, 30000–70000 and 17000–24000. The immunoprecipitates with the anti-(blood-group I) serum contained 11% of the radioactivity of the starting material; only 0.08% was precipitated with normal human serum. On electrophoresis, the anti-(blood-group I) immunoprecipitate showed a broad band with a peak at an apparent mol.wt. of 44000; the highest radioactivity (approx. 90% of that applied) was again at the dye front (Fig. 1*b*).

The ^{125}I γ-radioactivity profile of the solubilized ^{3}H/^{125}I-labelled stroma (Fig. 1*c*) showed a major peak at an approximate mol.wt. of 100000 (corresponding to Band 3), in addition to peaks at approx. 200000 (spectrin), 58000, 40000 (PAS 2) and at the dye front. The immunoprecipitate with the anti-(blood-group I) serum contained 2.1% of the radioactivity used; with normal human serum 0.12% of the radioactivity was precipitated. The ^{125}I γ-radioactivity profile of the anti-(blood-group I) immunoprecipitate (Fig. 1*d*) was very different from that seen by counting for ^{3}H (Fig. 1*b*). In agreement with earlier experiments (Childs *et al.*, 1978), the main ^{125}I-labelled blood-group-I-active component was a peak in the apparent mol.wt. range 90000–100000, corresponding to Band-3 protein. Two minor broad areas of radioactivity were observed in the apparent mol.wt. range 38000–70000 and 20000–31000. The ^{125}I radioactivity profile of the immunoprecipitate obtained with normal human serum (Fig. 1*d*, dotted line) showed only negligible radioactivity in the apparent mol.wt. range 90000–100000.

Polyacrylamide-gel-electrophoresis profiles of ^{3}H-labelled erythrocyte poly(glycosyl)ceramides and of their immunoprecipitates with anti-(blood-group I) serum

Figs. 2(*a*) and 2(*c*) show the radioactivity profiles of the ^{3}H-labelled poly(glycosyl)ceramide-rich fraction isolated by the method of Dejter-Juszynski *et al.* (1978) and the poly(glycosyl)ceramide subfraction 9A of Kościelak *et al.* (1976). In both cases a broad zone of radioactivity was observed migrating between apparent mol.wts. of 17000 and 75000. The immunoprecipitates with the anti-(blood-group I) serum contained 1.1 and 3% of the radioactivity, respectively, in the starting materials; with normal human serum 0.13 and 0.09% of the radioactivity, respectively, were precipitated. The electrophoretic patterns of the anti-(blood-group I) immunoprecipitates obtained with these two preparations (Figs. 2*b* and 2*d*) were similar. In each case, precipitating activity was confined to the slower-migrating compo-

nents of the original material, with a major peak at an apparent mol.wt. of 47000.

Discussion

These data show that different blood-group-I-active erythrocyte components are preferentially labelled by radioiodination or galactose oxidase/NaB^3H_4 treatment. The sensitivity of detection of antigenically active components in this type of experiment depends on the degree of incorporation of the radioisotope. This is evident with Band-3 protein, which has a carbohydrate content of only 5–8%; a higher specific radioactivity is likely to result from the incorporation of ^{125}I into the protein moiety rather than of ^3H into a limited number of accessible galactose residues. However, with highly glycosylated molecules of the membrane such as the low-molecular-weight glycolipids and poly(glycosyl)ceramides, some of which are known to have blood-group-I activity associated with terminal galactose residues (Feizi *et al.*, 1978; Niemann *et al.*, 1978; Watanabe *et al.*, 1979; Kościelak *et al.*, 1976), higher specific radioactivities and increased sensitivity of detection would be expected by the incorporation of ^3H.

The bands of radioactivity in the anti-(blood-group I) immunoprecipitates of the two poly(glycosyl)-ceramide preparations indicated that only a slowly migrating subpopulation of poly(glycosyl)ceramides is precipitable with anti-(blood-group I) antibody. There was a striking similarity between these two bands (Figs. 2*b* and 2*d*) and the diffusely migrating band observed with the immunoprecipitate of the ^3H-labelled solubilized membranes (Fig. 1*b*), suggesting that the latter may represent a subpopulation of poly(glycosyl)ceramides. It is possible that the minor, diffuse zone of radioactivity in the apparent mol.wt. range 38000–70000 observed with the immunoprecipitate counted for ^{125}I radioactivity (Fig. 1*d*) represents non-specific uptake of ^{125}I on to the double bonds (Welton & Aust, 1972) of the ceramide moiety of poly(glycosyl)ceramides.

Further experiments are required to investigate whether all the diffusely migrating ^3H-labelled blood-group-I-active components of the stroma in the apparent mol.wt. range 40000–70000 are poly(glycosyl)ceramides, and to determine their relationship to the blood-group-I-active membrane fraction isolated by Ebert *et al.* (1975) and to the diffusely migrating membrane components obtained with immune precipitates by using anti-globoside and anti-paragloboside antisera (Tonegawa & Hakomori, 1977). The minor area of radioactivity in the apparent mol.wt. range 20000–31000 observed with the ^{125}I-labelled immunoprecipitate (Fig. 1*d*) could represent the blood-group-I-active glycoprotein isolated from erythrocyte stroma by Hamaguchi & Cleve (1972). Thus, by these two labelling techniques it has been possible to obtain the spectrum of known blood-group-I-active components of erythrocyte membranes. In additional immunoprecipitation experiments using solubilized erythrocyte stroma prepared from intact cells labelled with ^3H by mild periodate oxidation of sialic acid residues followed by reduction with NaB^3H_4 (Gahmberg & Andersson, 1977), we were unable to demonstrate blood-group I activity with the electrophoretic mobilities of PAS bands 1, 2 and 3 (R. A. Childs, unpublished work). The periodate-oxidation conditions used were mild compared with those required for first-stage periodate oxidation and are unlikely to have destroyed terminal galactose residues associated with blood-group-I activity (Feizi *et al.*, 1971). Therefore the bands of radioactivity in the anti-(blood-group I) immunoprecipitates (Fig. 1*d*) are unlikely to represent PAS bands 1 and 2.

A limitation of immunoprecipitation (as well as affinity chromatography) experiments with these anti-blood-group antibodies is their dependence on the multivalent expression of antigens. This may in part account for the low percentage of precipitable blood-group-I activity detected in Band-3 protein (Childs *et al.*, 1978) and in the poly(glycosyl)ceramide fractions in the present studies. On the other hand, these findings are a reflection of the well-known microheterogeneity of oligosaccharide chains in glycoproteins and glycolipids. Even when glycoprotein preparations with strong blood-group-I activity are tested (before specific enrichment) in immunoprecipitation and affinity-chromatography experiments (for example, blood-group-I-active glycoprotein extracts from individual sheep gastric mucosae) only 25% of the macromolecules were specifically precipitable by anti-(blood-group I) antibody or retained by an anti-(blood-group I) immunoadsorbent column (Wood *et al.*, 1979). Nonetheless, experiments similar to those in the present studies with antisera specific for the blood-group-A, -B and-H antigenic determinants should give useful information on the still unresolved subject of the distribution of the major blood-group antigens on erythrocyte glycoproteins.

Note Added in Proof (Received 29 March 1979)

The occurrence of poly(glycosyl) chains on erythrocyte glycoproteins has been described (Krusius *et al.*, 1978; Järnefelt *et al.*, 1978); their terminal saccharide structures resemble those of poly(glycosyl)ceramides and contain sequences now known to be associated with blood-group-I activity.

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