The identification of specific Rhesus-polypeptide–blood-group-ABH-active-glycoprotein complexes in the human red-cell membrane

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1. RhD,c and E immune complexes isolated from ^3H - and ^{125}I -surface-radiolabelled and unlabelled intact human red cells were analysed by SDS/polyacrylamide-gel electrophoresis. 2. Apparent M_r values of 31 900 for RhD polypeptide and 33 100 for Rhc,E polypeptide were obtained under both reducing and non-reducing conditions. Glycosylation of RhD,c and E polypeptides was not detected. 3. RhD,c and E immune complexes also contain a glycoprotein component. RhD glycoprotein (apparent M_r 45 000–100 000) is distinct from Rhc,E glycoprotein(s) (apparent M_r 35 000–65 000). Rh (Rhesus) glycoprotein carbohydrate moieties are susceptible to endo- β -galactosidase digestion and carry blood-group-ABH determinants. This suggests the presence of polylactosaminoglycan-type structures. 4. Rh glycoproteins are not present in Rh immune complexes as a result of non-specific adsorption of membrane glycoproteins during the membrane-solubilization phase of immune-complex isolation because RhD immune complexes isolated from a 1:1 (v/v) mixture of Acde/cde and OcDE/cDE red cells do not contain blood-group-A-active glycoprotein. 5. Blood-group-A immune complexes isolated from group-A red cells of the appropriate Rh phenotypes contain the 31 900- and 33 100-apparent- M_r Rh polypeptides. 6. It was concluded from the above evidence that non-covalent Rh-glycoprotein–Rh-polypeptide complexes exist in the native red-cell membrane. 7. The 31 900- and 33 100-apparent- M_r Rh polypeptides are absent from blood-group-A immune complexes isolated from regulator type Rh_{null} cells (donor A.L.), but are replaced by a 33 800-apparent- M_r Rh_{null}-specific polypeptide (Rh_{null} polypeptide). It is suggested that Rh_{null} polypeptide is an aberrant product of the Rh gene complex.

INTRODUCTION

Despite the immunohaematological importance of the human Rh (Rhesus) blood-group system [1], little is known of the molecular composition of the individual antigens. In previous reports from this laboratory we have described integral red-cell membrane polypeptides which are involved in the expression of RhD,c and E antigen activities and suggested that RhD polypeptide is distinct from Rhc,E polypeptide(s) [2,3]. In addition, Rh polypeptides were shown to be distinct from those carrying the Fy (Duffy) and LW blood-group antigens [3]. The existence of RhD polypeptide has been independently confirmed [4,5,6] and data have been presented which suggests that, remarkably for a mammalian cell-surface protein, the RhD polypeptide is not glycosylated [4,5]. It has also been reported that RhD polypeptide interacts with the red-cell cytoskeleton [7,8,9] and that the RhD, and a related polypeptide that is bound by a monoclonal antibody recognizing a determinant in the Rh system, are both absent from red-cell membranes of the Rh_{null} regulator type [6]. These observations are consistent with the structural role for Rh polypeptides implied by the fragility of Rh_{null} red-cell membranes [1].

The organization of Rh polypeptides in the intact erythrocyte membrane is uncertain. Values of 60 000 and 174 000 for the apparent M_r of the RhD antigen in the intact red-cell membrane and values ranging from 7000 to 300 000 for the apparent M_r in solution have been reported [10–16]. This wide variation can be explained in part by assuming that the membrane components involved in Rh-antigen expression exist in oligomeric forms. In the present paper we show that the RhD polypeptide is distinct from Rhc,E polypeptide(s) and present evidence that the Rh polypeptides exist in the membrane complexed to glycoprotein components which carry blood-group-ABH determinants. We also investigate the possibility that membranes from red cells of the Rh_{null} regulator type contain an aberrant Rh polypeptide.

MATERIALS AND METHODS

Blood-group antibodies

Mouse monoclonal anti-A (IgG1 subtype) was a supernatant obtained from cultures of ES-16 (originally coded 131/3) hybridoma cell line [17]. Human polyclonal anti-D,c and -E sera were obtained from volunteer

Abbreviations used: sialoglycoprotein terminology is as described in [41]; α , sialoglycoprotein α (synonym glycophorin A); δ , sialoglycoprotein δ (synonym glycophorin B); PAGE, polyacrylamide-gel electrophoresis; BSA, bovine serum albumin; PBS-Triton, 0.01 M-sodium phosphate/0.15 M-NaCl/1% Triton X-100, pH 7.4; Rh, Rhesus; anti-A etc., anti-(blood-group A) serum (or antibody) etc.

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donors and adsorbed to remove anti-A and anti-B antibodies. Pregnancy-stimulated human polyclonal IgG anti-A and anti-B sera were obtained from volunteer group-O donors and adsorbed to remove group-B and group-A antibodies respectively.

Erythrocytes

Peripheral blood was obtained from volunteer donors. Red cells were washed six times (2000 g, 15 min) in PBS (0.01 M-sodium phosphate/0.15 M-NaCl, pH 7.4) and, if not used within 24 h of donation, were stored under liquid N₂ [18]. Frozen cells were recovered as described in [18]. ARh_{null} cells of the regulator type (donor A.L.) were generously given by Mr. J. Moulds and were stored under liquid N₂.

¹²⁵I-labelled protein A

A 1 mg portion of protein A (a gift from Dr. D. S. Pepper) was labelled with ¹²⁵I by the chloramine-T method [19]. The labelled protein was chromatographed on Sephadex G-25 equilibrated in PBS-Triton containing 1% BSA.

Immune-complex isolation

RhD,c and E immune complexes were isolated from 1 ml of packed red cells as previously described [2]. Briefly, radiolabelled or non-radiolabelled packed cells were incubated with blood-group antibody, washed, and membranes were isolated after hypo-osmotic lysis. The membranes were solubilized in PBS-Triton and soluble immune complexes isolated by adsorption to protein A-Sepharose CL-6B (Pharmacia). Immune complexes were eluted with 1% SDS and the eluate freeze-dried. Blood-group-A immune complexes were isolated as described above, except that packed cells were incubated with mouse monoclonal anti-A for 30 min at room temperature or with human polyclonal anti-A for 30 min at 37 °C. Continuous mixing for 15 s immediately after the addition of anti-A to cell suspensions and thereafter at approx. 5 min intervals was required to prevent the formation of a large single mass of agglutinated cells. Otherwise it is extremely difficult to lyse cells in the centre of a large agglutinate.

Cell-surface radiolabelling

Aliquots (1 ml) of packed washed cells were labelled with ¹²⁵I by the lactoperoxidase/glucose oxidase/Na¹²⁵I procedure as previously described [2]. Aliquots (1 ml) of red cells were surface-radiolabelled with ³H by using the galactose oxidase/NaB³H₄ or the periodate/NaB³H₄ procedures [20,21] with the following modifications. Cells were labelled by addition of 5 mCi of NaB³H₄ (5–10 Ci/mmol; Amersham International) for 30 min at room temperature. This was followed by a further 30 min incubation with unlabelled NaBH₄ (BDH) (3 mg/ml final concn.).

SDS/PAGE

Electrophoresis was performed on 7.5–15%-(w/v)-polyacrylamide gradient gels using a Laemmli buffer system [22] as previously described [2], except that resolution was increased by increasing gel length from 140 to 200 mm. A mixture containing ¹⁴C-labelled (Amersham International) proteins of 14300, 30000, 46000, 69000, 92500 and 100000 M_r was used to determine apparent-M_r values for red-cell membrane

components. All samples were reduced with 5% (v/v) 2-mercaptoethanol before electrophoresis.

Electroblotting to nitrocellulose and detection of blood-group-A determinants on immobilized components

Electrophoretic transfer was performed for 18 h at 10 V/cm in transfer buffer containing 0.192 M-glycine, 0.025 M-Tris and 20% (v/v) methanol, pH 8.2, maintained at approximately 10 °C by means of a heat exchanger. After transfer, the nitrocellulose membrane was washed in 200 ml of distilled water and then incubated for 30 min in 100 ml of PBS-Triton containing 4% BSA. The membrane was then washed in PBS-Triton and incubated for 1 h at room temperature in 100 ml of PBS-Triton containing 5 ml of mouse monoclonal anti-A from which protein aggregates had been removed by centrifugation (10 min, 2000 g). The membrane was washed four times in 200 ml of PBS-Triton and incubated for 1 h in 100 ml of PBS-Triton containing 4% BSA and 2 × 10⁷ c.p.m. of ¹²⁵I-labelled protein A taken from a stock solution which had been centrifuged immediately before use to remove aggregated material. The membrane was washed five times in PBS-Triton, twice in distilled water, air-dried and autoradiographed. In the above procedure all incubations and washes were performed with gentle orbital shaking.

Autoradiography

Detection of ¹²⁵I was performed as previously described [2]. ³H was detected by fluorography [23].

Pronase digestion of immune complexes

Immune complexes eluted from protein A-Sepharose CL-6B [2] were dialysed overnight against 0.1% SDS/0.0625 M-Tris/5 mM-EDTA/0.3% 2-mercaptoethanol, pH 6.8. Samples were heated at 100 °C for 2 min, cooled, and Pronase (Calbiochem) added to a final concentration of 50 µg/ml. Samples were incubated overnight at 37 °C, freeze-dried, and reconstituted in 0.12 ml of a solution containing 5% SDS/8 M-urea/5% 2-mercaptoethanol/0.001% Bromophenol Blue.

Endo-β-galactosidase treatment of ³H-labelled cells

Incubation with endo-β-galactosidase (*Escherichia freundii*; Miles Scientific) was performed as described in [24], except that 50% cell suspensions were incubated with endo-β-galactosidase at a final concentration of 20 munits/ml for 2 h at 37 °C.

RESULTS AND DISCUSSION

RhD polypeptide is distinct from Rhc,E polypeptides

Previous reports have identified integral red-cell membrane polypeptides associated with RhD,c and E antigen expression [2–6] and suggested that RhD polypeptide is not identical with Rhc,E polypeptide(s) [2]. The results in Fig. 1 confirm this non-identity. Rh immune complexes were isolated from ¹²⁵I-surface-radiolabelled red cells and analysed by SDS/PAGE. Apparent-M_r values of 31 900 were determined for RhD polypeptide (Fig. 1c) and 33 100 for Rhc,E polypeptide(s) (Figs. 1a and 1b). The discrepancy between these values and the previously reported apparent-M_r value of 28 500 for both RhD and Rhc,E polypeptides [2] can be attributed to the higher electrophoretic resolution achieved in the present study (see the Materials and

Fig. 1. Determination of the apparent M_r of RhD,c and E polypeptides

Rh immune complexes were isolated from ^{125}I -surface-radiolabelled cDE/cDE cells and separated by SDS/PAGE. (a) Rhc immune complexes; (b) RhE immune complexes; (c) RhD immune complexes. The dried gel was autoradiographed for 7 h.

methods section), which allowed resolution of RhD polypeptide from Rhc,E polypeptide(s) and the known dependency of Rh polypeptide apparent- M_r values on electrophoretic conditions [4].

The finding that at least two polypeptides are involved in the expression of Rh-antigen activity is supported by independent evidence. Firstly a monoclonal antibody which recognizes an undefined public antigen in the Rh system has been described [6,25]. This antibody binds to a 34000-apparent- M_r red-cell membrane polypeptide which is distinct from RhD polypeptide and which may be identical with the 33100-apparent- M_r Rhc,E polypeptide(s) described in the present study. Secondly, a two-locus theory for the genetic control of Rh-antigen expression has been proposed which is based on serological evidence and predicts that RhD-antigen expression is associated with a molecule different from Rhc and E antigens [26]. The above findings contrast with a recent report that all Rh antigens are located on the same molecule [27].

Rh immune complexes contain specific red-cell membrane glycoprotein components

RhD,c and E immune complexes were isolated from intact red cells radiolabelled with ^3H using either the galactose oxidase/ NaB^3H_4 or periodate/ NaB^3H_4 procedures and analysed by SDS/PAGE. ^3H was not incorporated into the RhD or Rhc,E polypeptides (Fig. 2). However, a previously unreported ^3H -labelled component, migrating as a diffuse band in the band 4.5 region, was readily detected in RhD,c and E immune complexes isolated from galactose oxidase/ NaB^3H_4 -labelled cells (Figs. 2d, 2f and 2h). Negligible ^3H incorporation was detected in immune complexes from periodate/ NaB^3H_4 -labelled cells (results not shown). Comparison of the 45000–100000-apparent- M_r range for the RhD glycosylated component (Fig. 2d) with the apparent- M_r range of 35000–65000 for the Rhc,E glycosylated component (Figs. 2f and 2h) suggests that at

least two distinct molecular populations have been identified with a wide range of electrophoretic mobility, suggesting heterogeneity of glycosylation.

Bands of ^{125}I -labelled components with the same apparent M_r as a proportion of the ^3H -labelled glycosylated components observed in Figs. 2(d), 2(f) and 2(h) were observed in RhD,c and E immune complexes isolated from ^{125}I -labelled cells (Figs. 2c, 2e and 2g). This suggests the possibility that the glycosylated components are glycoproteins. However, red-cell membrane macroglycolipids have an apparent- M_r range of 40000–70000 when analysed by SDS/PAGE [28]; thus it was important to determine the glycolipid or glycoprotein identity of the glycosylated components. To answer this question, ^3H -labelled RhD,c and E immune complexes were digested with Pronase and analysed by SDS/PAGE. A significant decrease in the apparent M_r of the RhD,c and E glycosylated components (Figs. 3f, 3d and 3e) was observed, thus indicating that the glycosylated components are glycoproteins. The nature of the bands migrating with apparent- M_r values of 120000 (Figs. 2c–2h) and 69000 (Figs. 2c, 2g and 2e) were not investigated. The latter may be Rh polypeptide dimer [5].

There is disagreement between a recent report [4,5] and the present study concerning the presence of a glycoprotein component in RhD immune complexes. This probably results from differences in procedures used for surface-radiolabelling of red cells with ^3H . Firstly we used a higher concentration of NaB^3H_4 . Secondly we found it was essential to reduce completely aldehyde groups remaining after red-cell tritiation by reduction with unlabelled NaBH_4 (see the Materials and methods section). Failure to include this step leads to a very high ^3H background in isolated Rh immune complexes (results not shown). This is probably due to formation of non-immune Schiff-base-linked-IgG-tritiated-membrane-glycoprotein complexes during incubation of red cells with blood-group-specific antisera. These complexes subsequently bind to protein A-Sepharose.

Action of endo- β -galactosidase on Rh glycoproteins and Rh polypeptides

Endo- β -galactosidase is known to cleave internal β 1-4 galactosidic links such as those found in $\text{GlcNAc}\beta$ 1-3Gal β 1-4Glc(or GlcNAc) structures present in band 3 and band 4.5 N-linked oligosaccharides [24]. The presence of similar structures in RhD,c and E glycoproteins is indicated by results of SDS/PAGE analysis of immune complexes isolated from ^3H -labelled red cells (Fig. 3). The apparent- M_r ranges of Rh glycoproteins from endo- β -galactosidase-treated and untreated cells were 43000–50000 and 45000–100000 respectively for RhD glycoprotein and 34000–42000 and 35000–60000 respectively for both Rhc and RhE glycoproteins. In contrast, endo- β -galactosidase did not alter the apparent M_r of the RhD polypeptide isolated from ^{125}I -labelled cells (results not shown). This finding, together with the observation that galactose oxidase and periodate-labelling procedures do not incorporate ^3H into Rh polypeptides indicates that RhD,c and E polypeptides are not glycosylated. Similar findings have been reported for RhD polypeptide [5].

Rh glycoproteins carry blood-group-ABH determinants

Blood-group-ABH-active structures are present on the non-reducing termini of the branched N-linked lactos-

Fig. 2. Detection of glycosylated red-cell membrane components in RhD,c and E immune complexes isolated from surface-radiolabelled red cells

Immune complexes were analysed by SDS/PAGE and the dried gels subjected to autoradiography. (a) Triton X-100-soluble fraction of membranes isolated from ^{125}I -surface-radiolabelled red cells; (b) Triton X-100-soluble fraction of membranes isolated from red cells radiolabelled with ^3H by the galactose oxidase/ NaB^3H_4 procedure; (c), (e), (g) and (i), Rh immune complexes isolated from ^{125}I -labelled cells; (d), (f), (h) and (j), immune complexes isolated from galactose oxidase/ NaB^3H_4 -labelled cells. (c) and (d), RhD immune complexes; (e) and (f), Rhc immune complexes; (g) and (h), RhE immune complexes; (i) and (j), negative control immune complexes isolated from cde/cde cells incubated with anti-D. Samples containing ^{125}I were run on gels separate from those containing ^3H . Autoradiography was performed for 3.5 days (c, e, g and i) and 21 days (b, d, f, h and j). The band labelled 'H' is an artefact caused by local mobility disturbances caused by the presence of IgG heavy (H) chain.

aminoglycan structures such as those found in band 4.5 and band 3 oligosaccharides. We investigated the possibility that RhD,c and E glycoproteins contain similar structures. Rhc,D and E immune complexes isolated from blood-group-A and -O red cells were analysed by SDS/PAGE and the separated components transferred to a nitrocellulose membrane by electroblotting. Blood-group-A determinants were detected by probing with monoclonal anti-A and ^{125}I -labelled protein A (Fig. 4). Blood-group-A activity was present only on the Rh glycoproteins isolated from blood-group-A individuals and not on Rh polypeptides. The latter observation is further evidence that Rh polypeptides are not glycosylated.

Rh-glycoprotein-Rh-polypeptide complexes exist in the native erythrocyte membrane

The difference in apparent- M_r values of both RhD glycoprotein and polypeptide compared with Rhc and E glycoprotein(s) and polypeptide(s) and the observation that apparent- M_r values of Rh polypeptides are unaffected by reduction with 2-mercaptoethanol (results not shown and [5]) argues that the interaction between Rh polypeptides and Rh glycoproteins in the native red-cell membrane is specific and non-covalent and is not due to non-specific adsorption of membrane glycoproteins by Rh polypeptides during the detergent-

solubilization stage of immune-complex isolation. Further evidence for specific association was obtained by analysing RhD immune complexes isolated from various mixtures of non-radiolabelled blood-group-OcDE/cDE, -Ocde/cde, -AcDE/cDE and -Acde/cde cells previously incubated with anti-D. If the RhD polypeptide associates non-specifically with glycoprotein during detergent solubilization of the red-cell membrane then blood-group-A-active glycoprotein will be found in RhD immune complexes isolated from a mixture of OcDE/cDE and Acde/cde red-cell membranes. The results in Fig. 4 clearly show that this does not occur.

The existence of Rh-polypeptide-Rh-glycoprotein complexes could explain some of the wide variation of apparent- M_r values for Rh antigens reported in the literature [10-16] if the complexes exist in oligomeric form. Their existence could also explain the report of an antibody which agglutinates only group-A RhD-positive cells [29]. It is possible that this antibody recognizes a neo-antigen formed by the close proximity of blood-group-A and RhD antigens in the RhD-glycoprotein-RhD-polypeptide complex. In addition, the apparently anomalous binding characteristics of RhD polypeptide to *Ricinus communis* lectin-Sepharose columns can be explained. The report [5] that *R. communis* lectin-Sepharose binds to RhD polypeptide present in unfractionated Triton X-100 extracts of red-cell membranes but not polypeptide purified by SDS/PAGE

Fig. 3. Effect of Pronase and endo- β -galactosidase on the glycosylated components present in Rh immune complexes

Immune complexes were isolated from cDE/cDE cells radiolabelled with ^3H by using the galactose oxidase/ NaB^3H_4 procedure and analysed by SDS/PAGE. Dried gels were subjected to autoradiography for 14 days. (a), (d) and (g), Rhc immune complexes; (b), (e) and (h), RhE immune complexes; (c), (f) and (i), RhD immune complexes. (d), (e) and (f), immune complexes were digested with Pronase before electrophoresis; (g), (h) and (i), immune complexes isolated from cells treated with endo- β -galactosidase immediately after radiolabelling. Immune complex samples in (a), (b) and (c) were treated as in (d), (e) and (f), except that Pronase was omitted.

can be explained, because SDS/PAGE separates the glycoprotein and polypeptide components of the RhD complex.

It is clear from the present study that both RhD polypeptide and RhD glycoprotein are molecular species different from their corresponding Rhc and RhE counterparts. This suggests that synthesis of Rh polypeptides and Rh glycoproteins are controlled by the Rh gene complex. The exact control mechanism is unknown. One intriguing possibility is that glycoprotein and polypeptide components of a specific Rh glycoprotein/Rh polypeptide complex are derived by post-translational cleavage of a glycosylated Rh precursor molecule. This hypothesis readily explains (i) the existence of non-covalent Rh-glycoprotein-Rh-polypeptide complexes in the red-cell membrane, (ii) why Rh polypeptides are not glycosylated and (iii) why the apparent M_r of RhD glycoprotein and polypeptide are different from their Rhc,E counterparts.

Detection of Rh polypeptides in blood-group-ABH immune complexes

Blood-group-A immune complexes were isolated from ^{125}I -labelled blood-group AcDE/cDE and -Acde/cde red cells and analysed by SDS/PAGE (Fig. 5). RhD polypeptide was detected in complexes from AcDE/cDE cells (Figs. 5e and 5h), but not from Acde/cde cells (Figs. 5f, 5i and 5j). The Rhc,E polypeptide(s) were detected in complexes from Acde/cde cells (Figs. 5f, 5i and 5j),

Fig. 4. Rh glycoproteins isolated from blood-group-A red cells carry blood-group-A antigenic determinants

Rh immune complexes were isolated from non-radio-labelled red cells selected from the following phenotypes: A+ (AcDE/cDE); A- (Acde/cde); O+ (OcDE/cDE); O- (Ocde/cde) and analysed by SDS/PAGE. Separated components were transferred to nitrocellulose by electroblotting. Blots were probed with monoclonal anti-A and ^{125}I -labelled protein A and autoradiographed for 18 h. (a) Rhc immune complexes from A+ cells; (b) RhD immune complexes from A+ cells; (c) RhE immune complexes from A+ cells; (d) Rhc immune complexes from O+ cells; (e) RhD immune complexes from O+ cells; (f), RhE immune complexes from O+ cells; (g) RhD immune complexes from a 1:1 (v/v) mixture of A- and O+ cells; (h) RhD complexes from a 1:1 (v/v) mixture of A+ and O- cells; (i) RhD immune complexes from A- cells; (j) RhD immune complexes from O- cells.

but were not resolved from RhD polypeptide in complexes from AcDE/cDE cells (Fig 5e and 5h). These results provide further evidence for the presence of Rh-polypeptide-blood-group-ABH-active-Rh-glycoprotein complexes in the native red-cell membrane. In addition, blood-group-A immune complexes contained band 4.5 and band 3 components, with the latter showing a higher apparent- M_r range compared with the bulk of band 3. Identical results were obtained when human polyclonal IgG anti-A and anti-B sera were used to isolate immune complexes from cells of appropriate ABH phenotype (results not shown).

The above data are the first report on the distribution of blood-group-ABH activity in red-cell membrane glycoproteins by isolation and analysis of immune complexes. The results agree with previous findings, which were based on lectin studies, that band 3 and band 4.5 are major carriers of blood-group-ABH activity [30,31,32]. However, previous reports disagree as to whether glycophorins A and B carry blood-group-ABH determinants. This is probably attributable to differences in the sugar specificity of the lectins used in these studies. We could not detect glycophorin A or B in blood-group-A immune complexes formed with either mouse monoclonal or human polyclonal antisera.

Investigation of the membrane defect of Rh_{null} erythrocytes

Studies with an impermeant maleimide reagent have

Fig. 5. Detection of Rh polypeptides in blood-group-A immune complexes.

Monoclonal anti-A was used to isolate immune complexes from ^{125}I -surface-radiolabelled blood-group-A cells. The complexes were analysed by SDS/PAGE and the dried gels were autoradiographed for various time periods to ensure accurate location of the positions of the major red-cell membrane proteins relative to the position of the Rh polypeptides. (a) and (d), Triton X-100-soluble fraction of ^{125}I -labelled Acde/cde red cells autoradiographed for 7 and 18 h respectively; (b), (e) and (h), blood-group-A immune complexes from AcDE/cDE red cells autoradiographed for 7, 18 and 36 h respectively; (c), (f), (i) and (j), blood-group-A immune complexes from Acde/cde red cells autoradiographed for 7, 18, 36 and 72 h respectively; (g) and (k), blood-group-A immune complexes from ARh_{null} red cells autoradiographed for 18 and 72 h respectively.

shown that components of 32000 and 34000 apparent M_r , identified in red cells of normal Rh phenotype, are absent from the membranes of Rh_{null} cells of the regulator type [6]. The 34000-apparent- M_r polypeptide was identified as the RhD polypeptide. The 32000-apparent- M_r polypeptide carries an Rh-gene-complex-specified determinant and is probably identical with the 33100-apparent- M_r Rhc,E polypeptide(s) described in the present study.

We investigated the red-cell membrane defect of Rh_{null} cells of the regulator type by an alternative procedure. It is possible that the regulator gene modifies normal transcription/translation of the Rh gene complex in such a way that aberrant Rh-polypeptide-Rh-glycoprotein complex(es) lacking Rh-antigen activity are produced. In this case the complex(es) would be present in blood-group-ABH immune complexes isolated from regulator-type Rh_{null} cells. Analysis of blood-group-A immune complexes isolated from group-A Rh_{null} regulator-type cells (Figs. 5g and 5k) clearly shows that the 31900-apparent- M_r RhD polypeptide and the 33100-apparent- M_r Rhc,E polypeptide(s) are absent. However, a 33800-apparent- M_r polypeptide was detected. This polypeptide (Rh_{null} polypeptide) was not detected in blood-group-A immune complexes isolated from AcDE/cDE and Acde/cde red cells and is therefore Rh_{null}-specific. It was also observed that the apparent M_r

values for the band 3 and band 4.5 components were consistently lower in blood-group-A immune complexes isolated from ARh_{null} cells compared with those from Acde/cde and AcDE/cDE cells. It is likely that these differences result from incomplete glycosylation that itself is attributable to anaemia-induced marrow stress.

The possibility can be discounted that Rh_{null} polypeptide is an incompletely glycosylated molecule, unrelated to Rh, which is present in red cells of normal Rh phenotypes in fully glycosylated form and which migrates as a diffuse component on SDS/PAGE. When blood-group-A immune complexes isolated from either periodate- or galactose oxidase/ NaB^3H_4 -labelled Rh_{null} red cells were analysed by SDS/PAGE and autoradiography, labelling of Rh_{null} polypeptide was not detected. In contrast, the band 3 and band 4.5 components, the latter appearing as a diffuse component in blood-group-A complexes isolated from normal cells, were readily labelled by both procedures (results not shown).

The exact nature of the structural differences between Rh_{null} polypeptide and RhD and Rhc,E polypeptides is not known. However, because Rh_{null} polypeptide was not detected in studies using a maleimide probe [6], it is probable that the free thiol group required for full expression of Rh-antigen activity [33,34,35] is either absent or inaccessible owing to the altered three-dimensional arrangement of Rh_{null} polypeptide.

Rh antigens are not located on band 3

Recent reports suggest that Rh-antigen activity is located on a subpopulation of band 3 [14,36–38]. The evidence obtained in this laboratory did not confirm these reports. Fig. 5 clearly shows that band 3 is a major component of blood-group-A immune complexes, but it is not detected in Rh immune complexes (Figs. 1 and 2). This argues against the possibility that band 3 is the native carrier of Rh-antigen activity but is degraded during the isolation of immune complexes. It is probable that Rh activity has been attributed to band 3 because of the binding of non-Rh-active immunoglobulin to receptors exposed on band 3 molecules [39,40] either by chemical treatment or detergent solubilization of membranes before incubation with antibody preparations. Such procedures were not used in the present study.

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REFERENCES

- Issitt, P. D. (1979) *Serology and Genetics of the Rhesus Blood-group System*, Montgomery Scientific Publications, Cincinnati
- Moore, S., Woodrow, C. F. & McClelland, D. B. L. (1982) *Nature (London)* **295**, 529–531
- Moore, S. (1983) in *Red Cell Membrane Glycoconjugates and Related Genetic Markers* (Cartron, J. P., Rouger, P. & Salmon, C., eds.), pp. 97–106, Librairie Arnette, Paris
- Gahmberg, C. G. (1982) *FEBS Lett.* **140**, 93–97
- Gahmberg, C. G. (1983) *EMBO J.* **2**, 223–227
- Ridgewell, K., Roberts, S. J., Tanner, M. J. A. & Anstee, D. J. (1983) *Biochem. J.* **213**, 267–269
- Gahmberg, C. G. & Karhi, K. K. (1984) *J. Immunol.* **133**, 334–337
- Ridgewell, K., Tanner, M. J. A. & Anstee, D. J. (1984) *FEBS Lett.* **174**, 7–10
- Paradis, G., Bazin, R. & Lemieux, R. (1986) *J. Immunol.* **137**, 240–247
- Green, F. A., Owens, N. A., Hui, H. L., Jung, C. Y. & Cuppoletti, J. (1983) *Mol. Immunol.* **20**, 361–365
- Folkerd, E. J., Ellory, J. C. & Hughes-Jones, N. C. (1977) *Immunochemistry* **14**, 529–531
- Abraham, C. V. & Bakerman, S. (1975) *Clin. Chim. Acta* **60**, 33–43
- Plapp, F. V., Kowalski, M. M., Tilzer, L., Brown, P. J., Evans, J. & Chiga, M. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 2964–2968
- Victoria, E. J., Mahan, L. C. & Masouredis, S. P. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2898–2902
- Evans, J. P., Sinor, L. T., Brown, P. J., Tilzer, L. L. & Plapp, F. V. (1982) *Mol. Immunol.* **19**, 671–675
- Lorusso, D. J. & Green, F. A. (1975) *Science* **188**, 66–67
- Moore, S., Scott, A., Micklem, L., Chirnside, A., James, K. & McClelland, D. B. L. (1984) *Dev. Biol. Stand.* **57**, 49–54
- Pepper, D. S. (1976) *Clin. Haematol.* **5**, 57–67
- Greenwood, F. C., Hunter, W. M. & Glover, J. S. (1963) *Biochem. J.* **89**, 114–123
- Mueller, T. J., Li, Y.-T. & Morrison, M. (1979) *J. Biol. Chem.* **254**, 8103–8106
- Gahmberg, C. G. & Andersson, L. C. (1977) *J. Biol. Chem.* **252**, 5888–5894
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88
- Fukuda, M. N., Fukuda, M. & Hakomori, S.-I. (1979) *J. Biol. Chem.* **254**, 5458–5465
- Merry, A. H., Thomson, E. E., Anstee, D. J. & Stratton, F. (1984) *Immunology* **51**, 793–800
- Tippett, P. (1986) *Ann. Hum. Genet.* **50**, 241–247
- Sinor, L. T., Brown, P. J., Evans, J. P. & Plapp, F. V. (1984) *Transfusion* **24**, 179–180
- Childs, R. A., Feizi, T. & Tonegawa, Y. (1979) *Biochem. J.* **181**, 533–538
- Ikin, E. W., Mourant, A. E. & Pugh, V. W. (1953) *Vox Sang.* **3**, 74–78
- Karhi, K. & Gahmberg, C. G. (1980) *Biochim. Biophys. Acta* **622**, 344–354
- Finne, J. (1980) *Eur. J. Biochem.* **104**, 181–189
- Viitala, J., Karhi, K. K., Gahmberg, C. G., Finne, J., Jarnefelt, J., Myllyla, G. & Krusius, T. (1981) *Eur. J. Biochem.* **113**, 259–265
- Abbott, R. E. & Schachter, D. (1976) *J. Biol. Chem.* **251**, 7176–7183
- Green, F. A. (1965) *Vox Sang.* **10**, 32–53
- Green, F. A. (1983) *Mol. Immunol.* **20**, 769–775
- Wallas, C. & Simon, B. (1984) *Transfusion* **24**, 425 (abstr.).
- Yokoi, T., Iwasa, M. & Sagisaka, K. (1983) *Tohoku J. Exp. Med.* **141**, 143–154
- Yokoi, T., Iwasa, M. & Sagisaka, K. (1983) *Tohoku J. Exp. Med.* **141**, 217–224
- Kay, M. M. B., Goodman, S. R., Sorensen, K., Whitfield, C. F., Wong, P., Zaki, L. & Rudloff, V. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1631–1635
- Victoria, E. J. & Mahan, L. C. (1981) *Mol. Immunol.* **18**, 699–704
- Anstee, D. J., Mawby, W. J. & Tanner, M. J. A. (1979) *Biochem. J.* **183**, 193–203

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