Human Monoclonal Antibody Against Rh(D) Antigen: Partial Characterization of the Rh(D) Polypeptide From Human Erythrocytes

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A human monoclonal anti-Rh(D) antibody produced by an Epstein-Barr virus (EBV)-transformed B-cell line (IgG1, clone H2DSD2) has been purified on protein A-Sepharose column and used for binding studies and immune precipitation of the blood group rhesus (Rh) antigens. Scatchard plot analyses show that the 125I-labeled antibody (iodo-gen procedure), binds to \(1.09 \times 10^5 \text{ mol/L}^{-1}\), \(0.43 \times 10^5 \text{ mol/L}^{-1}\), and \(0.32 \times 10^5 \text{ mol/L}^{-1}\) antigen sites on each D-/D-, RhR\(^{a}\) and RhR\(^{b}\) RBC, respectively, with an association constant of \(\approx 6 \times 10^8 \text{ mol/L}^{-1}\). Immune precipitation studies indicate also that the Rh(D) antigen of the Rh(D)-positive RBCs is carried by a 29 kDa polypeptide derived from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). No material could be precipitated from Rh(D)-negative or Rhnull RBCs. These results indicate that the monoclonal and the polyclonal human anti-Rh(D) behave similarly. A sample (Blo, presumed genotype R\(^{a}\)r or R\(^{b}\)R\(^{a}\)) showing an increased number of antigen sites (0.76 \(10^5 \text{ mol/L}^{-1}\)) and a high binding constant (5.7 \(10^6 \text{ mol/L}^{-1}\)) was used, as well as D--/D-- RBCs, for further purification of the 29-kd component. Extraction by Triton X-100 (0.1% to 5%) of the immune complexes formed between the membrane-bound Rh(D) antigens and the monoclonal antibody as well as a direct quantitative estimate of the 29-kd component, suggest that the Rh(D) polypeptide is loosely bound to the skeleton, since \(\approx 80\%\) can be solubilized from the membrane. In similar conditions, glycoprotein A showed a slight association with the Triton-insoluble residue, whereas glycoprotein B was easily and completely extracted. In contrast, both the minor RBC sialoglycoproteins, glycoprotein C and glycoprotein \(\gamma\), remained predominantly bound to the membrane skeleton. The purified Rh(D) polypeptide obtained from Blo. and D--/D-- RBCs by immunoprecipitation and preparative gel electrophoresis was homogenous as judged by SDS-PAGE. Amino acid composition indicated that the Rh(D) protein contained sulfhydryl groups which are essential for biological activity.  

**MATERIALS AND METHODS**

Materials. Reagent grade chemicals were used throughout. Protein A-Sepharose CL 4B (2 mg/mL of gel) was obtained from Pharmacia (Uppsala, Sweden) and *Staphylococcus aureus* absorbent was obtained from Miles-Yeda (Naperville). Iodo-gen (1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril) was bought from Pierce (Rockford, IL). \(^{125}\)Na (625 MBq/\(\mu\)g of iodine) was from the Radiocentre Chemical (Amersham, Bucks, England). Triton X-100 and bovine serum albumin (BSA) were purchased from Serva (Heidelberg, FRG) and from Sigma Chemicals (St Louis, respectively). Dibutylphthalate and bis-2-ethyl-hexylphthalate were obtained from Fluka (Busch, Switzerland). Red cells from donors of known Rh genotype (rr, R\(^{a}\)R\(^{a}\), R\(^{a}\)r, R\(^{a}\)^{b}\)) and from donor Blo. (probable genotype R\(^{b}\)R\(^{b}\) or R\(^{b}\)^{b}\)) were collected at the Centre National de Transfusion Sanguine (Paris, France). Rare RBCs (D--/D--, Gou.; Rhnull amorphic type, Fri.) were obtained from the Centre National de Référence sur les Groupes Sanguins (Paris) and were stored in liquid nitrogen until used.  

Purification of the human anti-Rh(D) monoclonal antibody. The human monoclonal anti-Rh(D) antibody [IgG1(A)] was produced by an EBV-transformed cell line (H\(_1\)D\(_1\)D\(_2\) clone) obtained from the B lymphocytes of an immunized donor. The monoclonal antibody was purified from the cell culture supernatant by protein A-Sepharose chromatography essentially as described. The tenfold concentrated supernatant (YM10 membrane, Amicon, Danvers,

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MA) was equilibrated in 100 mmol/L of sodium phosphate, 150 mmol/L of NaCl, pH 8.0, and was applied to a 10 mL of protein A-Sepharose column at room temperature and sequentially eluted at pH 6.5 (100 mmol/L of sodium phosphate, NaCl 150 mmol/L) and at pH 4.5 (100 mmol/L of sodium acetate buffer). The IgG molecules were finally eluted with 100 mmol/L of glycine, pH 2.8, and quickly neutralized using a 100 mL/Tris solution. Biological activity of the preparation was checked by a direct agglutination test performed with 50 mL of a 3% (vol/vol) papain-treated RBC suspension and 50 mL of the immunoglobulin fractions serially diluted in 150 mL/L of NaCl. Purity of the material obtained was controlled by PAGE in nonreducing conditions and gel permeation on a TSK G3000 SW column (LKB, Bromma, Sweden) developed at pH 7.2, 150 mmol/L of NaCl.

Binding of labeled anti-Rh(D) to human erythrocytes. The purified monoclonal anti-Rh(D) (500 mL, 1.5 mg/mL) was labeled by the iodogen procedure as described below, with 7.4 MBq 125I at room temperature. Free iodine was eliminated by gel filtration on a Sephadex G-25 column (15 x 1.5 cm) equilibrated in phosphate-buffered saline (PBS) (10 mmol/L of phosphate buffer, pH 7.2, 150 mmol/L of NaCl). The IgG fraction eluted in the void volume was used for the binding experiments. More than 95% of the material was trichloroacetic precipitable with a specific activity of 7.4 kBq/µg of protein. Binding studies to RBCs were carried out in 0.4-mL polypropylene microcentrifuge tubes essentially as described by Briles and colleagues. Fifty microliters of PBS containing 1.5 x 10⁶ cells was incubated, in duplicate, with 0.017 to 6 µg of 125I-labeled anti-Rh(D) and 2% (wt/vol) BSA for 1 hour at room temperature. The RBCs were then washed once in PBS containing 2% (wt/vol) BSA and further separated from unbound anti-Rh(D) molecules by the addition of 100 mL of oil (dibutylphthalate/bis-2-ethyl-hexylphthalate, 3/1, by vol) followed by centrifugation of the tubes. Bound radioactivity was determined by counting the pellet in a LKB γ counter (model 1274). Estimation of the number of antigen sites of each cell and the association constant for the anti-Rh(D) was calculated according to Scatchard.

Immune precipitation and purification of the Rh(D) protein. Immune precipitation of Rh(D) antigen from 125I-labeled Rh-positive RBC membranes was performed essentially according to Gahrberg. Fifty microliters of packed cells were suspended in 1 mL of PBS and labeled with 9.25 MBq 125I NaI at room temperature. Free iodine was eliminated by gel filtration on a Sephadex G-25 column (15 x 1.5 cm) equilibrated in phosphate-buffered saline (PBS) (10 mmol/L of phosphate buffer, pH 7.2, 150 mmol/L of NaCl). The IgG fraction eluted in the void volume was used for the binding experiments. More than 95% of the material was trichloroacetic precipitable with a specific activity of 7.4 kBq/µg of protein. Binding studies to RBCs were carried out in 0.4-mL polypropylene microcentrifuge tubes essentially as described by Briles and colleagues. Fifty microliters of PBS containing 1.5 x 10⁶ cells was incubated, in duplicate, with 0.017 to 6 µg of 125I-labeled anti-Rh(D) and 2% (wt/vol) BSA for 1 hour at room temperature. The RBCs were then washed once in PBS containing 2% (wt/vol) BSA and further separated from unbound anti-Rh(D) molecules by the addition of 100 mL of oil (dibutylphthalate/bis-2-ethyl-hexylphthalate, 3/1, by vol) followed by centrifugation of the tubes. Bound radioactivity was determined by counting the pellet in a LKB γ counter (model 1274). Estimation of the number of antigen sites of each cell and the association constant for the anti-Rh(D) was calculated according to Scatchard.
After 125I-labeling the monoclonal antibody retained its biological activity, and the binding to native Rh-positive RBCs occurred without agglutination and was saturable (Fig 1, inset). The binding data plotted according to Scatchard indicated that R' R' and R' R' erythrocytes carry 0.32 and 0.43 x 10^5 antigen sites, respectively (Fig 1). No binding occurred to Rhnull cells, which were devoid of Rh antigens. Dash erythrocytes (D--/D--), however, had two to three times more antigen sites (1.09 x 10^5) than did RBCs with a common phenotype. Erythrocytes Blo., which carry the D, c, E, and e antigens (probable genotype R'R') as deduced from the family study, not shown) had 0.76 x 10^5 antigen sites, a value intermediate between R'R' or R2R2 and D--/D-- erythrocytes. The association constants of the antibody to R'Blo.R' or R2R2 and D--/D-- erythrocytes were rather similar, ~0.6 x 10^5 mol/L^-1, but were 10 times lower than with Blo. erythrocytes (6.7 x 10^5 mol/L^-1). Because Blo. erythrocytes also have a larger number of Rh(D) antigens than do cells with common phenotype and were readily available (from one of us, C.B.), they were used for further characterization and purification of the Rh(D) polypeptide.

**Immune precipitation of the Rh(D) antigen.** The immune complexes formed between the monoclonal antibody and the Rh(D) polypeptide from 125I-labeled membranes were extracted by 5% Triton X-100 and isolated with a S. aureus absorbent. The components eluted from this absorbent are shown in Fig 2, after analysis by SDS-PAGE. A band with an apparent molecular mass of 29-kd was clearly identified in all RBC samples from Rh-positive individuals (Fig 2, lanes 2, 3, and 4), whereas no material could be identified from Rh-negative (Fig 2, lane 5) or Rhnull (Fig 2, lane 6) RBCs therefore indicating that this component represents presumably the Rh(D) polypeptide. Much more material was immunoprecipitated from D--/D-- and Blo. erythrocytes (Fig 2, lanes 2 and 3) than from R'R' RBCs (Fig 2, lane 4), correlating well with the mean number of Rh(D) antigen sites present on these cells. After gel electrophoresis, part of the radioactive material contained in the immune precipitates migrated as a light band of 58-kd (Fig 2, lanes 2 and 3) identified as the dimeric form of the 29-kd component following protein elution and reelectrophoresis (Fig 2, lanes 7 and 8).

**Comparison of detergent-solubilized properties of the Rh(D) polypeptide and of the RBC membrane sialoglycoproteins.** The distribution of the Rh(D) polypeptide...
between the Triton X-100 soluble and insoluble membrane extracts was first examined using 125I-labeled intact RBCs. The quantitative estimates were done by counting the radioactivity associated with the 29-kd band after SDS-PAGE of the Triton extracts. As shown in Table 1, the Rh(D) polypeptide was gradually solubilized from the membrane as the concentration of Triton X-100 increased. At low concentration of detergent, a significant part of the Rh(D) polypeptide was present in the insoluble residue. With 5% Triton X-100, however, ~71% of the Rh material was solubilized. Because these results differ from those of other researchers, the distribution of the Rh(D) antigen was also investigated following the binding of the 125I-labeled anti-Rh(D) to Rh-positive and Rh-negative erythrocytes. The results obtained using saturating amounts of antibody were in agreement with those obtained above since ~86% of the Rh(D) immune complexes were solubilized by 5% Triton X-100 (Table 1). When the suspension of immune complexes and detergent (1:3 by vol) were centrifuged immediately, however, without shaking for 30 minutes at 10 rpm, ~80% and 61% of the radioactivity was found in the pellet obtained from the 1% and 5% Triton X-100 extracts, respectively (not shown).

For comparison, the distribution of the main RBC membrane sialoglycoproteins were investigated in parallel by staining the detergent soluble and insoluble extracts, prepared from 125I-labeled membranes (including the shaking phase), with PAS after separation by SDS-PAGE (Table 1). More than 80% of glycophorin A was extracted by 1% Triton X-100 and almost 90% was removed from the membrane by 5% of detergent. Glycophorin A can be almost completely extracted by a high concentration of Triton X-100, in marked contrast with glycophorin C which remains firmly bound to the detergent insoluble residue even in the presence of 5% Triton X-100 (Table 1). Interestingly, glycophorin B was very easily solubilized from the membrane since ~70% of the glycoprotein was extracted by only 0.1% Triton X-100 and as much as 94.6% was removed by 1% of detergent (Table 1). Glycoprotein γ exhibited a behavior very similar to that of glycophorin C (Table 1), suggesting a strong association with the membrane skeleton.

Purification and amino acid analysis of the Rh(D) polypeptide. The Rh(D) antigen readily loses its biological activity following extraction from the RBC membrane. Therefore, the Rh(D) polypeptide was purified by immune precipitation of 125I-labeled erythrocyte membrane preparations by the monoclonal anti-Rh(D) antibody. The immune complexes extracted as above by 5% Triton X-100 were separated by preparative PAGE, and the Rh(D) polypeptide was isolated by excision and electroelution of the 29-kd component. From 10 ml of Rh-positive membranes (Blo.), ~1 nmol of the Rh(D) polypeptide was obtained in a pure form as deduced from analytical SDS-PAGE (not shown).

The purified preparation of Rh(D) polypeptide was virtually devoid of contaminating IgG (or IgG fragments), since there was no detectable band after electrophoretic transfer of the analytic SDS gel (5 µg loaded) to a nitrocellulose sheet treated by a sensitive immunostaining procedure, using a peroxidase-labeled anti-human IgG, capable of detecting as little as 10 ng of monoclonal anti-Rh(D) (not shown). In another control experiment, we were unable to detect any Coomassie-blue stainable material on the analytical SDS gel using the same purification protocol with erythrocyte membranes from an Rh-negative donor (not shown).

The Rh(D) polypeptide was purified by preparative gel electrophoresis by cutting out only the region around 29 kd. Assuming that Blo. erythrocytes carry 0.76 x 10^5 antigen sites (Fig 1), the purification yield of the Rh(D) polypeptide was ~7%. A similar purity and extraction yield was obtained with a preparation from D-/-/D-/- RBCs (not shown). Attempts to increase the purification yield by extracting the membrane skeleton with alkali before immune precipitation were unsuccessful. The amino acid composition of the Rh(D) polypeptide from Blo. erythrocytes is shown in Table 2. Two different preparations were analyzed. The small variations in amino acid composition were not unexpected since slightly different preparations were used. Therefore, the Rh(D) polypeptide was purified by immune precipitation of 125I-labeled erythrocyte membrane preparations by the monoclonal anti-Rh(D) antibody.

### Table 1. Solubilization of Glycoproteins and Rh Polypeptide From Human Erythrocyte Membranes by Triton X-100

<table>
<thead>
<tr>
<th>Detergent Concentration (%, wt/vol)</th>
<th>Fraction Analyzed</th>
<th>cpm Counting</th>
<th>Percentage of Material Solubilized Estimated by PAS Staining</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Rh (29 kd)*</td>
<td>[125I]anti-Rh(D)†</td>
</tr>
<tr>
<td>0.1</td>
<td>P</td>
<td>88</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>S</td>
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<td>19</td>
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<tr>
<td>0.5</td>
<td>P</td>
<td>64</td>
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<td></td>
<td>S</td>
<td>71</td>
<td>86</td>
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</table>

PAS, periodic acid-Schiff; GPA, GBP, GPC, glycophorins A, B, C; GPγ, glycoprotein γ (or component E) is also called glycophorin C by Forthmayr.
P, pellet; S, supernatant.

*Estimation of Rh(D) polypeptide from 125I-labeled RBC membrane components after sodium dodecylsulfate-polyacrylamide gel electrophoresis.
†Estimation of Rh(D) polypeptide using the monoclonal 125I-labeled anti-Rh(D) antibody.
observed were presumably attributable to the presence of residual glycine from the electrophoretic buffer (preparation 1) and of sodium azide (preparation 2), which may have altered the aspartate and histidine determinations. The results indicated that the Rh(D) polypeptide from these preparations contained several sulfhydryl residues and was composed of ~250 amino acid residues.

**DISCUSSION**

We studied the binding properties of a human monoclonal anti-Rh(D) antibody produced by an EBV-transformed lymphoblastoid cell line to Rh-positive and Rh-negative human erythrocytes. This antibody was used to characterize some properties of the Rh(D) antigens and to purify the Rh(D) polypeptide to homogeneity.

The number of Rh(D) antigen sites of R2R2, R'R' cells and of the rare D--/D-- erythrocytes, which do not carry Rh(D) antigens, thereby confirming the strict blood group specificity of the monoclonal antibody. Correlated well with the results reported by others using conventional polyclonal human antisera.

No significant binding occurred, however, onto Rh-negative or Rhnull erythrocytes, which do not carry Rh(D) antigens, thereby confirming the strict blood group specificity of the monoclonal antibody and its potential use as reagent for routine Rh typing. The equilibrium constant of the monoclonal antibody was also determined using RBCs of known Rh(D) antigen site density. A value close to 0.6 x 10^9 mol/L^-1 was calculated from the Scatchard plots drawn with R2R2, R'R', and D--/D-- RBCs. This is in good agreement with most estimations calculated using human IgG anti-Rh(D) present in the sera obtained from 21 women at the time of delivery; however, it is lower than the value found with the IgM monoclonal anti-Rh.

The monoclonal antibody used here binds with high affinity (6 x 10^6 mol/L^-1) to BlO. erythrocytes which, with their large number of Rh(D) antigen sites (0.76 x 10^5), were selected for the further characterization and purification of the Rh(D) polypeptide. The large increase of equilibrium constant noted with BlO. erythrocytes was not related to their high antigen content, since the monoclonal antibody binds with a lower affinity (0.6 x 10^4 mol/L^-1) to D--/D-- RBCs, which carry even more Rh(D) antigens (Fig 1). Other studies of the Rh haplotypes segregating in family BlO., using a large collection of human polyclonal antisera, should indicate whether the RBCs from these donors express an unusual form of Rh complex.

A 29-kd component could be specifically immunoprecipitated from all Rh-positive cells investigated, whereas no material was isolated from Rh-negative or Rhnull RBC membranes. This was again in agreement with previous studies carried out with polyclonal antisera. As suspected earlier, a dimeric form (58 kd) of the 29 kd was also identified on the gels, but no component of higher molecular mass was present in the immunoprecipitates. Using different approaches, we found that as much as 70% to 80% of the Rh(D) polypeptide can be extracted from Rh-positive RBCs by 5% Triton X-100 (Table 1) under conditions in which glycoporphins A and B were almost or completely extracted from the RBC membrane and glycoporphin C as well as glycoprotein γ were firmly bound to the Triton-insoluble residue. These results suggest that only a small proportion of the Rh(D) polypeptide interacts significantly with the membrane skeleton. Gahmberg and Karhi showed that 70% of the Rh(D) polypeptide was firmly anchored to the membrane skeleton. We also found that when the Triton X-100 extracts are prepared without shaking, 61% of the Rh(D) peptide is centrifuged with the 5% detergent insoluble residue. Up to 80% of this material was easily released after gentle shaking (10 rpm for 30 minutes), however, suggesting a loose association of the Rh(D) peptide with the membrane skeleton. These different results are clearly related to the method used for membrane solubilization, and only 60% to 72.7% of glycoporphin A antigen–antibody complexes were extracted by 5% Triton X-100 in the study reported above.

The Rh(D) polypeptide solubilized by 0.1% to 5% detergent could not be immunoprecipitated by the monoclonal antibody and did not bind to an affinity column prepared from the purified IgG monoclonal anti-Rh(D) antibody. The Rh(D) polypeptide was therefore purified on a large scale by a combination of immunoprecipitation and preparative PAGE using BlO. and D--/D-- RBC membrane preparations. The preparations obtained appeared homogeneous by usual criteria and had closely related amino acid compositions (Table 2), with a similar content of sulfhydryl groups. The yield of Rh(D) was, however, rather low (~7%), since the procedure could not be conducted with saturating amounts of anti-Rh(D) antibody. Indeed, from the binding studies reported above (Fig 1, inset), ~5 to 10 mg of purified anti-Rh(D) antibody was deemed required for each mL of packed RBCs used during the purification to maximize the yield. Using the present conditions, however, we obtained enough material for a partial characterization of the Rh(D) polypeptide and studies are now in progress to prepare a mouse monoclonal antibody against this protein, which would greatly facilitate its purification on a large scale for further biochemical analysis.
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