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Absence of two membrane proteins containing extracellular thiol groups in Rh_{null} human erythrocytes

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1. Rh_{null} human erythrocytes lack all the antigens of the Rhesus blood-group system and are associated with mild chronic haemolytic anaemia. These erythrocytes have an abnormal shape and increased osmotic fragility. 2. Labelling studies with the impermeant maleimide *N*-maleoylmethionine [^{35}S]sulphone show that Rh_{null} erythrocytes lack two extracellular thiol-group-containing membrane components of apparent mol.wts. 32000 and 34000. 3. Immunoprecipitation with mouse monoclonal antibody R6A (which reacts with all normal erythrocytes, but fails to react with Rh_{null} erythrocytes) specifically precipitates the 34000-mol.wt. component from normal erythrocytes. Similar studies with human anti-Rh(D) serum shows that this antibody reacts with the 32000-mol.wt. component. 4. The results suggest that the R6A-binding polypeptide and the Rh(D) polypeptide may be involved in the maintenance of the shape and viability of the human erythrocyte.

The occurrence of human erythrocytes with a total lack of all antigens within the Rhesus blood-group system (denoted Rh_{null} erythrocytes) was first described by Vos *et al.* (1961). This condition was subsequently shown to be associated with the presence of mild chronic haemolytic anaemia. Rh_{null} erythrocytes have an abnormal shape (stomatocytosis) and also have an increased osmotic fragility (Sturgeon, 1970). The cause of the defect giving rise to these abnormalities is unknown.

Until recently the nature of the membrane components which carry the major antigens of the Rhesus blood-group system was unclear. However, Moore *et al.* (1982) and Gahmberg (1982) have reported that the antigens C, D and E of this blood-group system are located on a membrane component that migrates as a band of apparent mol.wt. 29000 on SDS/polyacrylamide-gel electrophoresis. The C and D antigenic determinants are lost on treatment with *N*-ethylmaleimide (Green, 1965). The extracellular location of a thiol group necessary for the activity of the D antigen was further suggested by Abbott & Schachter (1976), who reported that an impermeant maleimide (glutathione maleimide) inactivated the D antigen activity of intact erytrocytes.

A comparison of the extracellular thiol-group-containing membrane components of normal and Rh_{null} erythrocytes should help to define the nature of the defect in the Rh_{null} cell. We describe here the results of an investigation of the alterations that occur in Rh_{null} erythrocytes using the impermeant maleimide *N*-maleoylmethionine [^{35}S]sulphone ([^{35}S]MMS) as a probe for membrane components containing extracellular thiol groups. We have also studies the membrane component recognized by the monoclonal antibody LICR/LON R6A (R6A). This antibody reacts with all normal human erythrocytes but fails to react with Rh_{null} erythrocytes (Anstee & Edwards, 1982). Our results show that two extracellular thiol-group-containing components with apparent mol. wts. 32000 and 34000 are absent from the Rh_{null} erythrocyte membrane.

Abbreviations used: LICR/LON: Ludwig Institute of Cancer Research, London; [^{35}S]MMS: *N*-maleoylmethionine [^{35}S]sulphone; SDS, sodium dodecyl sulphate; IgG, immunoglobulin G.

Materials and methods

Mouse monoclonal antibody R6A (Edwards, 1980) was used as unprocessed ascitic fluid. Human anti-D serum was kindly provided by Dr. S. Moore (South East Regional Blood Transfusion Service, The Royal Infirmary, Edinburgh, Scotland, U.K.). Rh_{null} erythrocytes from donor A. L. were kindly provided by Mr. J. Moulds (Gamma Biologicals,

Houston, TX, U.S.A.), and Rh_{null} erythrocytes from donor Y. T. were kindly provided by Dr. P. Harden (Red Cross Blood Transfusion Service, Brisbane, Australia). The erythrocytes were used within 9 days of the blood being drawn. Rh-positive control erythrocyte samples of comparable age were available from the South Western Regional Blood Transfusion Service, Bristol, U.K.

[^{35}S]MMS was prepared as described by Roberts *et al.* (1982) at a specific radioactivity of 0.3–0.4 Ci/mmol. Erythrocytes were washed twice with 10 vol. of 0.15 M-NaCl and once with 0.1 M-sodium phosphate buffer, pH 8.0, before labelling. Intact erythrocytes (100 μl of washed, packed cells) were labelled with 50 μCi of [^{35}S]MMS (Roberts *et al.*, 1982). The volume was made up to 120 μl with 0.1 M-sodium phosphate buffer, pH 8.0. After incubation for 1 h at room temperature the cells were washed twice with 10 vol. of 0.1 M-sodium phosphate buffer, pH 8.0, containing 5 mM-2-mercaptoethanol and lysed with 10 vol. of 5 mM-sodium phosphate buffer, pH 8.0 (lysis buffer). The membranes were washed once with 10 vol. of lysis buffer, stripped of peripheral proteins with 10 vol. of 0.1 M-NaOH containing 5 mM-2-mercaptoethanol, and then washed twice in lysis buffer.

A 1 ml portion of washed packed control erythrocytes was labelled with Na^{125}I (Amersham International; sp. radioactivity 13.4 mCi/μg) by using lactoperoxidase as described by Moore *et al.* (1982). Membranes were prepared and SDS/polyacrylamide-gel electrophoresis carried out as described by Ridgwell *et al.* (1983). Immunoprecipitation of radiolabelled components from 1 ml of ^{125}I-labelled cells was as described by Ridgwell *et al.* (1983). When human anti-D serum was used, the additional incubation was with rabbit anti-human IgG instead of rabbit anti-mouse IgG.

Results

The extracellular thiol-group-containing membrane components of intact normal and Rh_{null} erythrocytes were labelled with the impermeant maleimide [^{35}S]MMS. The membranes were stripped of peripheral proteins and the labelled components separated by SDS/polyacrylamide-gel electrophoresis. Fig. 1 shows that the Rh_{null} membranes clearly lack two closely spaced bands of apparent mol.wts. 32000 and 34000. These closely spaced bands correspond to the band denoted 'δ' by Abbott & Schachter (1976), who did not resolve them under their conditions of SDS/polyacrylamide-gel electrophoresis, although Roberts *et al.* (1982) have previously suggested that the band is a doublet.

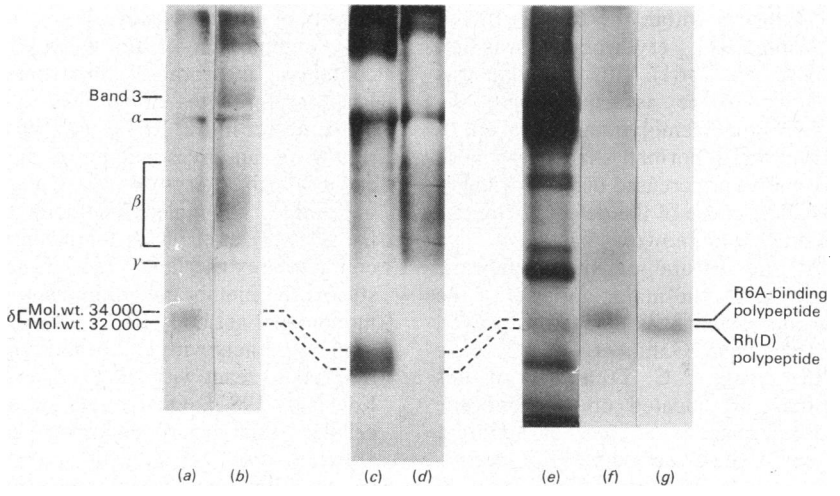

Fig. 1. *Labelling of intact normal and RH_{null} erythrocytes with [^{35}S]MMS and immunoprecipitation of membrane components from ^{125}I-labelled intact normal erythrocytes*

(*a–d*) Autoradiographs of solubilized, NaOH-stripped [^{35}S]MMS-labelled erythrocyte membranes from: (*a*) normal erythrocytes, (*b*) A. L. Rh_{null} erythrocytes, (*c*) normal erythocytes and (*d*) Y. T. Rh_{null} erythrocytes. (*e–g*) Autoradiographs of: (*e*) solubilized membranes from ^{125}I-labelled normal erythrocytes, (*f*) components from ^{125}I-labelled normal erythrocyte membranes precipitated by monoclonal antibody R6A, and (*g*) components from ^{125}I-labelled normal erythrocyte membranes precipitated by polyclonal anti-D serum. Much less radioactivity was obtained in components precipitated by R6A than in components precipitated by anti-D serum. Therefore the portion of the gel shown in (*f*) was exposed for 3 weeks, whereas the portion of the gel shown in (*g*) was exposed for 1 week. Gels (*a–b*), (*c–d*) and (*e–g*) were run on separate occasions, therefore the mobility of the bands are slightly different in each case.

The monoclonal antibody R6A reacts with normal human erythrocytes, but fails to react with Rh_{null} erythrocytes (Anstee & Edwards, 1982). Immunoprecipitation, with R6A, of membrane components from intact erythrocytes radioiodinated with lactoperoxidase gave the results shown in Fig. 1. A component of apparent mol. wt. 34000 was immunoprecipitated from normal erythrocytes by R6A. This component has the same electrophoretic mobility as the larger of the two components in the doublet that is found in [^{35}S]MMS-labelled normal erythrocytes, but is absent from Rh_{null} erythrocytes. Fig. 1 also shows that a polyclonal anti-D antiserum immunoprecipitates a component of apparent mol.wt. 32000 from normal erythrocytes. This component has the same electrophoretic mobility as the smaller of the two components in the doublet found in [^{35}S]MMS-labelled erythrocytes and which is absent from Rh_{null} erythrocytes.

Discussion

Rh_{null} erythrocytes appear to lack two components carrying extracellular thiol groups and of apparent mol.wts. 32000 and 34000. The component of apparent mol.wt. 34000 (R6A-binding polypeptide, Fig. 1) is precipitated by monoclonal antibody R6A, whereas anti-D antibody precipitates the component of apparent mol.wt. 32000 [Rh(D) polypeptide]. The latter component corresponds to the Rh(D) polypeptide identified by Moore *et al.* (1982) and Gahmberg (1982).

Monoclonal antibody R6A was reported to immunoprecipitate specifically the human erythrocyte anion-transport protein Band 3 (Edwards, 1980) Our results clearly show that this antibody precipitates a component of apparent mol. wt. 34000 and not Band 3 (mol. wt. 100000). The absence of the R6A-binding protein in Rh_{null} cells (which have apparently normal Band 3) supports this conclusion. Our conditions of immunoprecipitation are quite different from those used by Edwards (1980), who immunoprecipitated from solubilized radioiodinated erythrocyte ghosts. The anion-transport protein is the most abundant erythrocyte membrane component and is also heavily labelled when erythrocytes are radioiodinated using lactoperoxidase. In our hands, immunoprecipitation under these conditions gives rise to a very high non-specific background precipitation of Band 3. Since the R6A binding polypeptide is a minor labelled component, prolonged autoradiography is necessary to detect it. The presence of contaminating Band 3 could thus result in the misassignment of the specificity of this antibody.

The distinction between Rh(D)-negative and Rh_{null} cells should be emphasized. Whereas Rh_{null} cells appear to lack all the antigens of the Rh system and fail to react with R6A, Rh(D)-negative cells lack only the D antigen and retain reactivity with the monoclonal antibody R6A. The D antigen is likely to be a polymorphic determinant on the Rh(D) polypeptide, whereas the monomorphic determinant recognized by R6A is located on the R6A-binding polypeptide. The absence of both these polypeptides from Rh_{null} erythrocytes suggests that they may be functionally related. The nature of this function is not known, but is presumably associated with the defective morphology and osmotic fragility of the cells. Clearly, the possible involvement of these two proteins in erythrocyte morphology and viability merits further investigation.

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