Biochem. J. (1986) 234, 649–652 (Printed in Great Britain)

Identification and partial characterization of the human erythrocyte membrane component(s) that express the antigens of the LW blood-group system

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1. Rh_{null} human erythrocytes lack the antigens of the Rhesus blood-group system, have an abnormal shape, have an increased osmotic fragility, and are associated with mild chronic haemolytic anaemia. Rh_{null} erythrocytes also lack all antigens of the LW blood-group system, but the functional significance of this deficiency is unknown. 2. We have identified, by immunoblotting with two mouse monoclonal antibodies (BS46 and BS56), the LW-active component(s) in normal human erythrocytes as a broad band of M_r 37000–47000 on SDS/polyacrylamide-gel electrophoresis. 3. Treatment of intact human erythrocytes with endoglycosidase F preparation destroyed the epitopes recognized by antibodies BS46 and BS56, suggesting that one or more *N*-glycosidically linked oligosaccharides are required for the formation of the LW antigens. 4. Estimation of the number of LW antigen sites per erythrocyte by using radioiodinated purified antibody BS46 gave average values of 4400 molecules/cell for Rh(D)-positive adult erythrocytes and 2835 molecules/cell for Rh(D)-negative adult erythrocytes. 5. Like the Rh(D) polypeptide, the LW polypeptide(s) is (are) associated with the cytoskeleton of normal erythrocytes. 6. These results suggest the possibility that the absence of the LW polypeptide may also contribute to the functional and/or morphological abnormalities of Rh_{null} erythrocytes.

INTRODUCTION

The LW blood-group system comprises two antigens (LW^a and LW^b) regulated by allelic genes and defined by the antithetical antibodies anti-LW^a and anti-LW^b respectively (Sistonen *et al.*, 1983). Two individuals are known (Mrs. Big. and her brother) who have the phenotype LW(a−b−) but normal antigens of the Rhesus blood-group system. Individuals of the rare Rh_{null} phenotype lack all antigens of the Rhesus blood-group system and are also LW(a−b−). The reason for this phenotypic association between antigens of two genetically independent blood-group systems is unclear. This phenotypic association is further demonstrated by the well-documented observation that antisera containing anti-LW specificities react more strongly with adult erythrocytes when they express the Rhesus (D) antigen than when they do not. Anti-LW sera may therefore appear to be anti-Rh(D) in specificity when tested with adult erythrocytes. Little is known with regard to the molecular nature of either the Rh(D) antigen or the LW antigens; however, it has been shown that the Rh(D) antigen is associated with a transmembrane polypeptide of M_r approx. 30000 (Moore *et al.*, 1982; Gahmberg, 1982; Ridgwell *et al.*, 1983), that this polypeptide is associated with the erythrocyte cytoskeleton (Ridgwell *et al.*, 1984; Gahmberg & Karlin, 1984), and that the polypeptide may lack carbohydrate (Gahmberg, 1983). Individuals with the rare Rh_{null} phenotype appear to lack the Rh(D) polypeptide and also another polypeptide (the R6A polypeptide) of M_r 34000 (Ridgwell *et al.*, 1983). The only information concerning the nature of the membrane component(s) expressing LW antigens is that of Moore (1983), who identified a membrane component of M_r 40000 by immunoprecipitation with the anti-LW^{ab}-containing serum of the LW(a−b−) individual Mrs. Big. More recently, the production of two monoclonal antibodies with specificity anti-LW^{ab} has been described by Sonneborn *et al.* (1984). In the present paper we have used these monoclonal antibodies to define further the nature and abundance of the erythrocyte membrane component(s) expressing the LW antigens and to show that the LW antigens are susceptible to endo-β-*N*-acetylglucosaminidase F preparations from *Flavobacterium meningosepticum*.

MATERIALS AND METHODS

Erythrocytes from the LW(a−b−) donor Mrs. Big. were kindly supplied by Dr. B. P. L. Moore, Canadian Red Cross Blood Transfusion Service, Toronto, Ont., Canada. Erythrocytes from the Rh_{null} donor (Y.T.) were kindly supplied by Dr. P. Harden, Red Cross Blood Transfusion Service, Brisbane. Queensland, Australia. Human monoclonal anti-D culture supernatant BB1 was kindly provided by Dr B. Kumpel, U.K. Transplant Service, Bristol U.K. Human polyclonal anti-D serum

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was a gift from Dr. C. Hodson, Lancaster Blood Transfusion Service, Lancaster, U.K.

Monoclonal antibodies BS46 and BS56 have been described previously (Sonneborn *et al.*, 1984). Antibody BS46 (IgG1; Sonneborn *et al.*, 1984) was purified from ascitic fluid by affinity chromatography on a rabbit anti-(mouse IgG) antibodies column and labelled with ^{125}I for estimation of antigen sites as described previously (Merry *et al.*, 1986).

Endo-β-*N*-acetylglucosaminidase F was prepared from cultures of *Flavobacterium meningosepticum* as described by Elder & Alexander (1982). This preparation contains both endo-β-*N*-acetylglucosamindase F and a peptidyl *N*-glycanase activity (Plummer *et al.*, 1984). Endo-β-galactosidase was prepared from cultures of *Flavobacterium keratolyticus* as described by Kitamikado *et al.* (1981). *F. keratolyticus* (strain I.F.O. 14087) was obtained from the type culture collection at Osaka, Japan. Treatment of erythrocytes with endoglycosidase was as follows. Erythrocytes were washed twice with 0.15 M-NaCl, then twice with 0.1125 M-NaCl/0.0497 M-EDTA/25 mM-sodium phosphate buffer, pH 6.0. The final wash solution contained 2 mM-phenylmethanesulphonyl fluoride. After incubation with endoglycosidase overnight at 37 °C in a final wash solution, the cells were washed four times in 0.15 M-NaCl before serological tests or preparation of erythrocyte ghosts.

Treatment of intact erythrocytes with neuraminidase, with trypsin and with Pronase has been described previously (Anstee *et al.*, 1979; Tanner *et al.*, 1980; Merry *et al.*, 1986). Treatment of intact erythrocytes with dithiothreitol was as described by Konighaus & Holland (1984).

The methods for preparation of erythrocyte membranes and SDS/polyacrylamide-gel electrophoresis have been described previously (Anstee *et al.*, 1984). Erythrocyte cytoskeletons were prepared by the method of Mueller & Morrison (1981).

Immunoblotting was carried out as follows. SDS/polyacrylamide-gel electrophoresis of erythrocyte membranes was in a slab containing 10% (w/v) acrylamide with an overlay of 3% acrylamide, with the buffer system of Laemmli (1970). The separated membrane components in unstained gels were electrophoretically transferred on to nitrocellulose membrane (Bio-Rad Trans-Blot Transfer Medium). Transfer was carried out at 4 °C for 2.5 h at 250 mA in a Trans-Blot apparatus (Bio-Rad) in a transfer buffer consisting of 25 mM-Tris/192 mM-glycine/20% (w/v) methanol. After transfer, the nitrocellulose membrane was immersed in 0.3% Tween/phosphate-buffered saline (Batteiger *et al.*, 1982) for 30 min at room temperature. The membrane was cut into strips corresponding in width to the individual membrane samples applied to the original polyacrylamide gel, and each strip was incubated for 1 h at room temperature in 0.3% Tween/phosphate-buffered saline containing 5% (v/v) normal rabbit serum before incubation for 18 h at 4 °C with undiluted culture supernatant containing monoclonal antibody. The strips were then washed twice in 0.3% Tween/phosphate-buffered saline (5 min in each wash) and incubated (1 h at room temperature) with a 1/200 dilution of horseradish peroxidase-conjugated rabbit anti-(mouse IgG) antibodies (Dakopatts, Copenhagen, Denmark) in 0.3% Tween/phosphate-buffered saline containing 5% normal rabbit serum. After incubation, the strips were washed twice in 0.3% Tween/phosphate-buffered saline for 5 min, immersed for approx. 10 s in a substrate solution consisting of 3,3′-diaminobenzidine (0.6 mg/ml; Sigma Chemical Co., Poole, Dorset) in 0.1 M-Tris/HCl buffer, pH 7.6, containing 0.1% (v/v) H_2O_2, immersed in distilled water for 5 min and air-dried.

RESULTS AND DISCUSSION

Identification of erythrocyte membrane components reacting with monoclonal antibodies BS46 and BS56

Immunoblotting of the electrophoretically separated components of normal erythrocyte ghosts solubilized under non-reducing conditions with either antibody BS46 or antibody BS56 identified a broad region of staining of M_r 37000–47000 (Fig. 1, tracks a, e and h). Within this broad staining region at least two distinct bands were discernible. No binding of either monoclonal antibody was seen when erythrocyte ghosts from either individual of the rare Rh_{null} phenotype (Y.T.) (Fig. 1, track b) or the LW(a−b−) donor Mrs. Big. (Fig. 1, track g) were similarly treated.

These results are similar to those of Moore (1983), who obtained an apparent M_r of 40000 for the LW component by immunoprecipitation from ^{125}I-labelled erythrocytes with a human anti-LW^{ab} serum (Big. serum).

Immunoblotting of neuraminidase-treated normal erythrocyte ghosts (Fig. 1, track d) resulted in a sharpening of the LW-antigen-containing region, M_r 36000–43000, with a decrease in the staining in the higher-M_r region and a slight shift in the apparent M_r at the front of the band. This suggests that the broad staining observed in normal cells is, to some extent, due to heterogeneity in sialylation. Cytoskeleton preparations from normal erythrocytes showed a pattern of binding identical with that observed for normal erythrocyte ghosts (Fig. 1, track c).

Antibodies BS46 and BS56 do not react with Pronase-treated normal erythrocytes (Lomas & Tippett, 1985). Consistent with this, immunoblotting analysis of the electrophoretically separated components of Pronase-treated normal erythrocytes showed no antibody binding (Fig. 1, track f).

Antibodies BS46 and BS56 also bound, though more weakly, to immunoblots of the electrophoretically separated components of erythrocyte ghosts solubilized under reducing conditions (results not shown). Konighaus & Holland (1984) reported that treatment of intact erythrocytes with 100 mM-dithiothreitol for 30 min at 37 °C destroys LW-antigen reactivity, and we have confirmed this observation. This result suggests the involvement of intrachain disulphide bonding in the LW glycoprotein, and may explain the weaker reactions we observe on immunoblotting with samples prepared in the presence of 2-mercaptoethanol.

Effect of endoglycosidases on the LW components

Treatment of normal erythrocytes with an endo-β-*N*-acetylglucosaminidase F preparation for 16 h at pH 6.0 completely destroyed the reactivity of antibodies BS46 and BS56 with these cells, as assessed by the indirect antiglobulin method. This enzyme had no effect on the Rh(D) antigen, as assessed by agglutination with human monoclonal anti-D culture supernatant derived from a lymphoblastoid cell line (Doyle *et al.*, 1985). Endo-β-

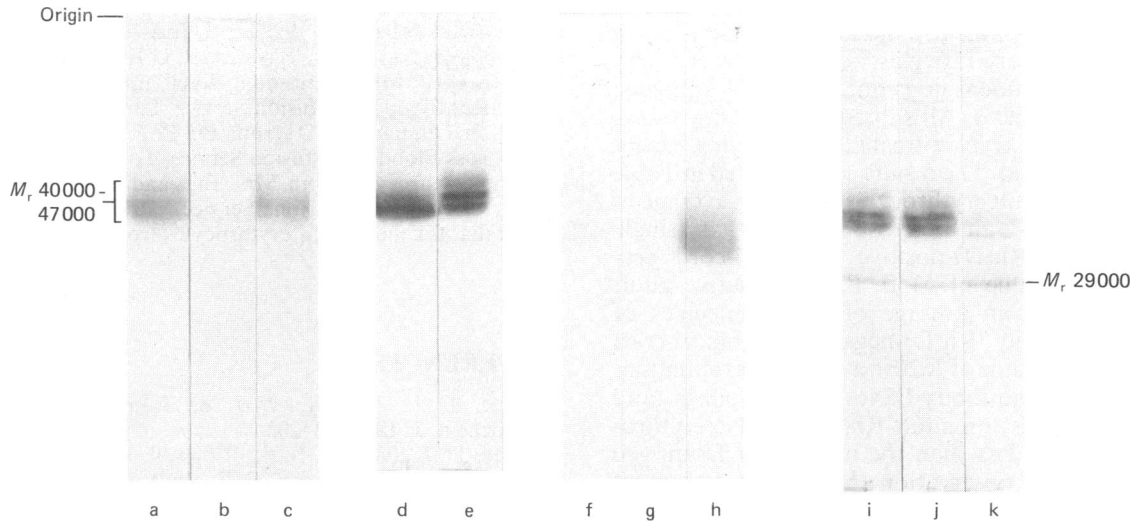

Fig. 1. Identification of LW polypeptide(s) by immunoblotting with monoclonal antibodies BS46 and BS56

Tracks a–c, samples solubilized under non-reducing conditions and immunoblotted with antibody BS46 (antibody BS56 gave identical results): a, normal ghosts; b, Rh_{null} erythrocyte ghosts (donor Y.T.); c, cytoskeletons from normal erythrocyte ghosts. Tracks d–m, samples solubilized under non-reducing conditions and immunoblotted with antibody BS56: d, neuraminidase-treated normal erythrocyte ghosts; e, normal erythrocyte ghosts; f, Pronase-treated normal erythrocyte ghosts; g, LW(a−b−) erythrocyte ghosts (donor Mrs. Big.); h, normal erythrocyte ghosts; i, normal erythrocyte ghosts incubated as for endoglycosidase treatment but without enzyme; j, endo-β-galactosidase-treated normal erythrocyte ghosts; k, endo-β-*N*-acetylglucosaminidase F-treated normal erythrocyte ghosts. The Figure is compiled from three different immunoblots. Tracks a, b and c are from one, tracks d, e, i, j and k from another, and tracks, f, g and h from the third. The band of M_r 29000 (tracks, i, j and k) does not result from specific binding antibody of BS56, since monoclonal antibodies directed at different erythrocyte membrane components also bound to this component.

Table 1. Estimation of the number of binding sites for monoclonal antibody BS46 on normal and Rh_{null} erythrocytes

Values for cDE-positive cells are the means of three determinations, and for ce-positive (D-negative) cells are the means of two determinations. D antigen sites were determined with ^{125}I-labelled polyclonal anti-D antibodies. Abbreviation: N.D., not determined.

Rhesus phenotype of cells	Sites/cell for Monoclonal antibody BS46	Sites/cell for Human anti-D
cDE	4400 (3990–4970)	23750
ce (D-negative)	2835 (2650–3020)	900
Rh(D)-positive (cord)*	5150	N.D.
Rh(D)-negative (cord)*	3620	N.D.
Rh_{null}*	380	N.D.
cDE (papain-treated)	4530	N.D.
cDE (Pronase-treated)	940	N.D.

*Trypsin-treated cells.

galactosidase treatment under the same conditions had no effect on the agglutination of normal erythrocytes by either monoclonal antibody. Immunoblotting demonstrated that endo-β-*N*-acetylglucosaminidase F substantially decreased the binding of the monoclonal antibodies (Fig. 1, track k) whereas endo-β-galactosidase treatment did not result in any detectable alteration in the LW-antigen band. These results suggest that the LW polypeptide(s) do(es) not contain an *N*-glycosidically linked oligosaccharide of the poly-*N*-acetyl-lactosaminyl type similar to that found on band 3 (erythroglycan; Fukuda & Fukuda, 1981), but more probably contain one or more of the smaller *N*-glycosidically linked oligosaccharides similar to those found on the major sialoglycoprotein α (syn: glycophorin A; Yoshima *et al.*, 1980). It is clear that these oligosaccharides are essential for the structure of the epitope recognized by antibodies BS46 and BS56 on the LW polypeptides.

Estimation of the number of LW antigen sites per erythrocyte

Monoclonal antibody BS46 (IgG1; Sonneborn *et al.*, 1984) was purified and iodinated, and the numbers of molecules of antibody bound to different erythrocyte samples were determined by Scatchard analysis. Preliminary experiments revealed a high level of non-specific

binding of labelled antibody BS46 to Rh_{null} erythrocytes (which lack the LW antigens). This non-specific binding was removed if the erythrocytes were first treated with trypsin (trypsin treatment does not effect LW antigens; Lomas & Tippett, 1985). All subsequent binding assays were performed with trypsin-treated erythrocytes, except where otherwise stated. The results are presented in Table 1. Rh(D)-positive adult erythrocytes bound an average of 4400 molecules of antibody BS46, and a single determination on Rh(D)-positive umbilical-cord erythrocytes gave a value of 5150. Rh(D)-negative adult erythrocytes bound an average of 2835 molecules of antibody BS46, and Rh(D)-negative umbilical-cord erythrocytes gave a value of 3620 in a single determination. The number of antibody-BS46-binding sites (and presumably LW sites) on adult Rh(D)-positive erythrocytes was markedly less than the number of D-antigen sites (Table 1). The observation that papain treatment of erythrocytes has little effect on the LW antigens whereas Pronase treatment inactivates the antigen (Lomas & Tippett, 1985) was confirmed in the binding assay (Table 1).

The results presented here clearly show that the LW antigens are associated with different membrane components from those that express the Rh(D) antigen and the R6A polypeptide, both of which are also absent from Rh_{null} erythrocytes (Ridgwell *et al.*, 1983).

The nature of the association between the LW antigens and the antigens of the Rh blood-group system is not clear. Gahmberg (1983) has suggested that the Rh(D) polypeptide is not glycosylated. The requirement of the LW antigen for an *N*-glycosidically linked oligosaccharide suggests the possibility that the LW polypeptide(s) is a glycosylated form of an Rh-related polypeptide. Unfortunately the loss of the antigen activity after endo-β-*N*-acetylglucosamindase F treatment prevents us from identifying the deglycosylated products and determining whether they have a similar M_r to that of the Rh(D) and/or R6A polypeptide. Like the Rh(D) polypeptide, the LW polypeptide(s) is associated with the cytoskeleton of normal erythrocytes. Rh_{null} erythrocytes have functional and morphological abnormalities that have been related to the absence of the Rh(D) polypeptide and the R6A polypeptide (Ridgwell *et al.*, 1983). These results suggest the possibility that the absence of the LW polypeptides may also contribute to the functional and/or morphological abnormalities in Rh_{null} erythrocytes. The individuals with LW (a−b−) phenotype but normal Rhesus blood-group antigens do not have abnormalities of the type found in individuals with Rh_{null} syndrome. This suggests that the loss of the LW antigenic determinants is not sufficient to cause the abnormalities associated with Rh_{null} syndrome, and this may be because loss of LW antigen from the erythrocytes of Mrs. Big. is due to defective glycosylation of the LW polypeptide rather than to the lack of the polypeptide.

Received 19 July 1985/19 September 1985; accepted 11 November 1985

We thank Dr. J. H. Elder, Research Institute of Scripps Clinic, La Jolla, CA 92037, U.S.A., for the culture of *F. meningosepticum*, Dr. B. Kumpel, U.K. Transplant Service, Bristol, U.K., for human monoclonal anti-D, Dr. C. Hodson, Lancaster Blood Transfusion Service, Lancaster, U.K., for the polyclonal human anti-D serum, Dr. B. P. L. Moore, Canadian Red Cross Blood Transfusion Service, Toronto, Ont., Canada, for the erythrocytes from Mrs. Big. and Dr. P. Harden, Red Cross Blood Transfusion Service, Brisbane, Queensland, Australia, for the Rh_{null} erythrocytes from donor Y.T.

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