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Murine monoclonal antibodies against a unique determinant of erythrocytes, related to Rh and U antigens: expression on normal and malignant erythrocyte precursors and Rh_{null} red cells

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Summary. Three murine monoclonal antibodies (Mabs) MB-2D10, LA-18.18 and LA-23.40 were prepared. They reacted with red cells of all common and most rare blood-group phenotypes, with the exception of those of the $Rh_{null}U$ negative and $Rh_{mod}U$ negative phenotypes. So far, only a single example of an alloantibody (Duclos or anti-Rh38) of a similar specificity has been found.

Serological studies indicated that the Mabs were probably not directed against an antigenic determinant of Rh polypeptides, the LW^{ab} glycoprotein or glycophorin B, all structures absent from or aberrantly expressed on Rh_{null} red cells. The antigen was found to be erythrocyte-specific, and was also present on pro-erythroblasts, erythroblasts and malignant erythroblastoid cells but not on erythroid progenitors in the bone marrow.

The Mabs were found to block each other in an immune rosette method and are thus probably directed against the same epitope or against neighbouring epitopes on the same structure.

In immunochemical studies, MB-2D10 precipitated the 30–32 kDa Rh polypeptides from red cell membranes and a protein or proteins which formed diffuse and overlapping bands in SDS–polyacrylamide gel electrophoresis, with M_rs of 40–200 kDa (probably the Rh-related glycoproteins). Under certain experimental conditions glycophorin B appeared to be coprecipitated.

The 2D10 structure, detected by the Mabs, seems to be part of a complex of proteins and/or glycoproteins, which includes Rh polypeptides, the LW^{ab} glycoprotein and glycoproteins recognized by various Mabs with Rh-related specificities. In the red cell membrane, the complex may be associated with glycophorin B.

Murine monoclonal antibodies (Mabs) with so-called Rh-related specificities are mostly identified by their failure to react with red cells of the rare phenotype Rh_{null}. Murine Mabs seem unable to recognize common DCE phenotype differences, but appear to be directed against public Rh antigens or related structures. For example, R6A (Anstee & Edwards, 1982) behaves as anti-Rh29 by reacting with all red cells except those of the rare Rh-null phenotype. BS-58 (Sonneborn *et al*, 1986) behaves as anti-Rh17 in tests against papain-treated cells in reacting with all red cells except those of Rh-null and -D- phenotypes. Three Mabs (Sonneborn *et al*, 1984; Oliviera *et al*, 1984) detect the LW^{ab} antigen or the *LW* core structure. Hence, such antibodies are not useful for Rh phenotyping, but are useful tools to study the biochemical structure and the expression of the Rh or Rh-related antigens on cells other than red cells.

In this report we describe the production and characteriza-

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tion of murine Mabs with an interesting 'new' Rh-related (and U-related) specificity. The antibodies did not react with $Rh_{null}U$ negative and $Rh_{mod}U$ negative red cells, but reacted with $Rh_{null}U$ positive and $Rh_{mod}U$ positive cells, as well as with cells of other rare Rh variants and common Rh phenotypes. This antibody specificity was similar to the human alloantibody Duclos (anti-Rh38) (Habibi *et al*, 1978). The antibodies were red cell specific and reacted also with immature erythroid cells but not with progenitor cells. The antibody precipitated not only the 30–32 kDa Rh polypeptide from red cells, but also a protein or proteins with M_rs of 40–200 kDa.

MATERIALS AND METHODS

Preparation of the Mabs. Swiss Webster mice were immunized three times (weekly intervals) with 1–2 μg of a Rh-D-polypeptide preparation, suspended in complete Freund's adjuvant, by intraperitoneal injection. The D-polypeptides were prepared as follows: 60 ml of 0 cDE/cDE red cells (platelet- and leucocyte-depleted by cotton-wool filtration and washing) was incubated with 40 ml of a polyclonal IgG-anti-D (CLB IgG anti-D) diluted in 90 ml phosphate-buffered saline (PBS) containing bovine serum albumin (BSA) 1% (w/v) for 1 h at 37°C. After washing once in PBS-BSA 1% w/v and three times in PBS, the cells were solubilized in 420 ml of a mixture of 0·5 mM Na_2 EDTA, 0·5 mM phenylmethylsulfonyl fluoride (PMSF), 20 μg/ml trypsin inhibitor, 0·1% (v/v) Tween-20, 0·5 mM β-mercaptoethanol and 1% (v/v) NP-40 in PBS by incubation for 1 h at 4°C. The lysate was then centrifuged for 30 min at 0–4°C in a Sorvall SW34 rotor at 12000 rpm. The supernatant was absorbed with 17 ml Sepharose protein A 10% (w/v) in solubilization mixture, followed by washing three times in this mixture, acid elution (in 8 ml of 1% (v/v) NP-40 in 0·2 M glycine, pH 2·5) of D-polypeptides for 10 min at 0°C and neutralization of the eluate (2 M Tris). The amount of eluted protein was determined spectrophotometrically (absorbance at 280 nm).

Our protocol for hybridization and cloning of Mab-producing cells has been described in detail elsewhere (Astaldi *et al*, 1980). Antibody-producing clones were detected in an ELISA with peroxidase goat anti-mouse Ig on red cell ghosts and then by an indirect antiglobulin test on intact red cells with unlabelled goat anti-mouse Ig. In this way, four stable red cell Mab-producing cell clones were obtained, one of which (code MB-2D10) was the subject of our study. Two other Mabs (LA-18.18 and LA-23.40) with similar properties to MB-2D10 were accidentally produced as a by-product of an experiment, in which we obtained CD10 Mabs by the immunization of mice with cALLA-positive blasts derived from the blood of a patient with ALL. The clones were detected with intact red cells using ELISA. All three Mabs were IgG1. For further studies, ascitic fluids were prepared by intraperitoneal injection of mice, after pristane priming.

Other monoclonal antibodies. CLB-ery-1 (anti-glycophorin A), CLB-ery-2 (anti-P), CLB-HLA-DR-2 (MB-CIA2) (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands), R6A (anti-Rh29) from D. Anstee (National Blood Transfusion Service, Bristol, U.K.), OSK-2 (anti-Rh29) (Osaka Red Cross Blood Centre, Osaka, Japan), BS-46 (anti-LW^{ab}) (Biotest Serum Institute, Offenbach, F.R.G.), AL-774 (anti-Rh17) (Ortho Diagnostic Systems, Raritan, N.J.), Bac 9 (anti-D), H2-D5 (anti-D), RaE11 (anti-c) and MCG8 (anti-E) (Centre National de Transfusion Sanguine, Paris, France).

Erythrocyte serology. The Mabs were tested with red cells in the direct agglutination test, the indirect antiglobulin test with goat or rabbit anti-mouse immunoglobulin serum and after enzyme treatment (papain-cystein, ficin, bromelin, trypsin, chymotrypsin, pronase, neuraminidase) by standard methods.

The antibodies were tested against a large panel of red cells, many recovered from storage in liquid nitrogen or in glycerol at −30°C. Included were all common ABO, Ii and Rh phenotypes, as well as rare phenotypes such as $r''^{G}r$, $hr^{S}-$, $hr^{B}-$, D− −, D. ., Rh_{null} (both of the amorph and the regulator type), Rh_{mod}, LW(a+b−), LW(a−b−), P_1^k, P_2^k, Lu(b−), Lu(a−b−) dominant type, K_o, Fy(a−b−), Wr(a+b−), En(a−), S−s−U−, M^kM^k, O_h, adult i, Vel− and LKE−.

Cellular expression of the antigen detected by the Mabs. This was studied on cells in suspension fixed in 1% paraformaldehyde by means of immunofluorescence with FITC-goat anti-mouse Ig. Immunofluorescence was measured by flow cytofluorometry on an EPICS-C or a FACS machine. Various isolated peripheral blood cells (platelets, granulocytes, basophils, monocytes, lymphocytes), unseparated bone-marrow cells, human fetal liver cells, cultured human myeloid (HL-60, ML-1, KG-1, U937), proerythroblastic (K562, HEL), T-lymphoid (HSB, CCRF-CEM, Peer), B- and pre-B lymphoid (Daudi, Raji, SB, Reh, NALM-16, Ros), neuroblastic (CHP 212) cell-line cells were tested, as well as suspensions of trypsinized cultured normal human fibroblasts and cord vein endothelial cells.

Expression of the antigen on colony-forming cells. This was tested in bone marrow precursor cell cultures, as previously described (von dem Borne *et al*, 1986). Ficoll–Isopaque purified mononuclear cells from normal human bone marrow were used. Cluster and colony formation (CFU-GM, CFU-E, BFU-E) were scored after 7 and 14 d of culture. Reactivity of the Mabs with precursor cells was tested by means of an immunorosette depletion method.

Immunorosette depletion. The method has been described in detail elsewhere (Slaper-Cortenbach *et al*, 1989). It is based on the finding of Lansdorp *et al* (1986) that, by the addition of a rat anti-mouse IgG1 Mab (code RB 5·3) in a mixture of two mouse Mabs of different specificity of the IgG1 class, bispecific tetrameric immune complexes are formed together with monospecific complexes which contain only one of the antibodies.

Mixtures were prepared of one part of the Mabs MB-2D10, LA-18.18 or LA-23.40 and half a part of IgG1 anti-human glycophorin-A Mab (CLB-ery-1). A double amount of the monoclonal rat anti-mouse IgG1 antibody was then added. After incubation (1 h at room temperature, overnight at 4°C), the mixture was tested for agglutination in different dilutions against human red cells, pretreated with chymotrypsin, in a suspension of 5×10^8/ml of red cells. Chymotrypsin treatment, necessary to render the red cells less agglutinable for

the Mabs under study (see later), was performed by incubating equal parts of a washed red cell suspension (50% v/v) and chymotrypsin solution (8 mg/ml in PBS) (30 min at 37°C). The highest subagglutinating dose was then added to the red cell suspension, incubated for 1 h at room temperature and then washed. The suspension of red cells, sensitized with the tetrameric complexes, was then mixed with mononuclear bone-marrow cells in a ratio of 10:1. The mixture was centrifuged and incubated (15 min at room temperature). After resuspension, the rosetted antigen-positive bone-marrow cells were separated from nonrosetted antigen-negative cells by Ficoll–Isopaque gradient centrifugation ($\delta = 1{\cdot}077$ g/cm^3). As a positive control, IgG1 HLA-DR Mab (MB-CIA2) was used. Sterile conditions were applied throughout.

Immunochemical studies on red cells. These were performed exclusively with MB-2D10 Mab, or IgG1 purified from it by ion-exchange chromatography using a Mono-Q column (Pharmacia Fine Chemicals, Uppsala, Sweden), equilibrated with Tris-HCl buffer 20 mM, pH 7·2, and eluted by a linear gradient of NaCl (0–1 M) in the same buffer.

(A) *Immunoprecipitation analysis.* Two methods were used for immunoprecipitation analyses.

Method 1 (presensitization method): Immunoprecipitation of MB-2D10 antigen from ^{125}I-labelled red cell membrane preparations was carried out by the procedure of Gahmberg (1983) with some modifications, as described elsewhere (Bloy *et al*, 1987, 1988a). Since MB-2D10 appeared to bind insufficiently to protein A, the immune complexes from sensitized membranes were extracted at 4°C with 1% (w/v) Triton X-100 and 200 μl of clear supernatant (centrifuged for 30 min at 37000 *g*) was further incubated with 30 μl of rabbit anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, Pa.) for 1 h at 4°C. The mixture containing the immune complexes was then absorbed with 50 μl of packed protein-A Sepharose beads (Pharmacia) for 1 h at 4°C. The beads were washed on a sucrose cushion and the bound radioactive material was eluted for analysis by SDS–polyacrylamide gel electrophoresis under non-reducing conditions in a discontinuous buffer system using a 10% (w/v) polyacrylamide-separating gel. Radioactive components were detected by autoradiography of the dried gels.

Method 2 (prelysis method). Immunoprecipitations were also performed from 1% (w/v) Triton X-100 lysates of red cell membrane preparations incubated with a preformed complex of an IgG-purified fraction of MB-2D120 and rabbit-anti-mouse IgG according to Borst *et al* (1982). The precipitates were washed on a sucrose cushion and analysed by SDS–polyacrylamide gel electrophoresis as described above.

(B) *Antigen site determination.* Purified IgG1 MB-2D10 labelled with ^{125}I was used. The number of MB-2D10 epitopes on red cells of various Rh and SsU phenotypes was calculated from Scatchard plots according to published protocols (Bloy *et al*, 1988a).

(C) *Competitive radiobinding assays.* The binding of purified ^{125}I-labelled antibodies (anti-D, c, E and MB-2D10) to cDE/cDE red cells was estimated in the presence of varying amounts of unlabelled antibodies as competitors, according to a method described elsewhere (Hughes Jones *et al*, 1988).

(D) *Two-dimensional chymotryptic peptide map analysis.* The applied techniques have been described in detail elsewhere (Blanchard *et al*, 1988; Saboori *et al*, 1988). In brief, the 30–32 kDa Rh polypeptides were immunopurified with MB-2D10 and human monoclonal anti-D antibody H2-D5 as well as purified chemically, from the red cells of the same O cDE/cDE donor. The isolated polypeptides were labelled with ^{125}I using chloramine-T and purified by SDS–polyacrylamide gel electrophoresis. The gel slices containing the radioactive 30–32 kDa bands were digested with chymotrypsin. This was followed by the analysis of the released iodopeptide (80% of the input of radioactivity) by electrophoresis and chromatography on cellulose plates and autoradiography.

RESULTS

Red cell serology

In tests on red cells of common phenotype, MB-2D10 agglutinated saline suspensions (titre 1:100), but it reacted much more strongly in the indirect antiglobulin technique (IAGT) (titre exceeding 1:10^5). Agglutination was also enhanced by albumin, some enzymes and polyethylene glycol. The antibody reacted with all red cells lacking high frequency antigens, with the exception of some Rh_{null} cells. This led to further investigations of MB-2D10 with other Rh_{null} and Rh_{mod} samples. It became clear that MB-2D10 reacted with Rh_{null} and Rh_{mod} red cells only if the cells expressed the U-antigen. Similar results were obtained with the Duclos antiserum ($P < 0{\cdot}05$) (Table I).

Other rare Rh-variant cells (D− −, D. .), Fy(a−b−) cells, LW(a−b−) cells and U-S-s-cells reacted strongly with MB-2D10. Hence, it appeared that MB-2D10 behaved in a similar way to the Duclos antibody (Habibi *et al*, 1978). Surprisingly, M^kM^k cells and En(a−) cells were agglutinated even more strongly than cells of common Rh phenotype.

With the two other Mabs, LA-18.18 and LA-23.40, identical serological results were obtained, although these antibodies were studied somewhat less extensively.

Effects of enzyme treatment of red cells

Red cells of common Rh and U phenotypes were treated with enzymes and tested with the Mabs in agglutination and antiglobulin tests (Table II). With MB-2D10 (prediluted 1/5000) direct agglutination was detected with papain-, trypsin-, ficin- and neuraminidase-treated red cells. Reactivity in the antiglobulin test was enhanced by treatment with papain and trypsin. Treatment with chymotrypsin dimi-

Table I. Result of testing MB-2D10 and Duclos antiserum on Rh_{null} and Rh_{mod} samples from unrelated donors

Phenotype	Samples	MB-2D10	Duclos
Rh_{null} U+	2	+	+
Rh_{null} U−	9	−	−
Rh_{mod} U+	1	+	+
Rh_{mod} U−	1	−	−

Table II. Results of enzyme treatment of red cells on serological reactivity with MB-2D10*

Enzyme	Agglutination test	Antiglobulin test
None	0	59
Papain	67	74
Trypsin	62	74
Chymotrypsin	2	40
Pronase	0	0
Bromelin	0	0
Ficin	54	61
Neuraminidase	35	57

* Doubling dilutions of MB-2D10, prediluted 1/5000 in buffered saline containing 2% (w/v) BSA, antiglobulin test with rabbit anti-mouse Ig serum. The titration score is shown. The red cells were from a donor with a common Rh and U phenotype (O cDE/cDE, U+).

nished reactivity, although this was variable in different experiments. The MB-2D10 reaction was completely abolished by pronase and bromelin treatment.

With the other two Mabs, identical results were obtained. Red cells of the $Rh_{null}U$ positive phenotype reacted as strongly with the three Mabs (in terms of titre and score) as red cells of common Rh phenotypes. Moreover, the effect of enzyme treatment on the reactivity of these cells was similar to that with red cells of common Rh phenotypes.

Expression of the antigen on other cell types

The Mabs did not react with nonerythroid peripheral blood cells (platelets, granulocytes, monocytes, basophils, lymphocytes) nor with cultured fibroblasts or endothelial cells as tested by immunofluorescence. On four different bone-marrow cell samples (one from a patient with pernicious anaemia) apart from red cells, a variable percentage (18–50%) of nucleated cells reacted. These appeared to be erythroblastoid (pro-erythroblasts and erythroblasts) from immunoenzyme stains of cells sedimented on slides (alkaline phosphatase method).

Also bone marrow cells of many patients with all kinds of acute and chronic haematological malignancies were tested (leukaemia, lymphoma). Only normal erythroblasts or malignant erythroblastoid cells from patients with AML-M6 or CML blast crisis (three cases each) showed a positive reaction in immunofluorescence and/or immunoenzyme stains (data not shown).

With cultured human leukaemic or carcinomatous cell-line cells (lymphoid, myeloid, neuroblastoid), no reaction was found with the exception of the cells of pro-erythroblastic cell lines (K562, HEL), which reacted strongly (Table III) and cells of the B-lymphoblastoid line Raji, which reacted more weakly and in a lower percentage (21%) (not shown).

The Mabs reacted with a much higher percentage of K562 and HEL cell line cells than other Mabs against Rh or Rh-related antigens (Table III). It was similar to that obtained with anti-glycophorin A. There was virtually no expression of globoside on either cell line, as tested with anti-P.

Table III. Results of testing MB-2D10 and other red cell-reactive Mabs by flow cytofluorometry with proerythroblastic cell-line cells

		K562		HEL	
Specificity	Code	%	I	%	I
Duclos-like	MB-2D10	96	156	96	148
Anti-Rh29	OSK-2	12	106	34	107
Anti-LW^{ab}	BS-46	12	105	45	101
Anti-Rh17	AL-774	12	107	45	105
Anti-D*	Bac 9	8	135	24	150
Anti-E*	MCG 8	5	142	25	132
Anti-glycophorin A	CLB-ery 1	85	154	88	144
Anti-P	CLB-ery 2	13	113	7	130
Anti-cat-hair-allergen	Negative contr.	4	100	5	124

* Human IgG Mabs, used in conjunction with FITC anti-human Ig. Results expressed as percentage (%) positive cells. Fluorescence intensity (I) of the positively reacting cells is also shown.

Five human fetal liver cell suspensions (15–19 weeks gestation) were also studied. These cells, consisting of about 80% proerythroblasts and erythroblasts, express glycophorin A and globoside (P-antigen) strongly (von dem Borne *et al*, 1987). A similar percentage of positive cells was found with the Mabs under investigation.

To study the reactivity of the three Mabs with red cell progenitors, erythroid colony formation of normal bone marrow was measured, with and without immune rosette depletion for antigen-positive cells. As depicted in Table IV, depletion did not affect either CFU-E formation or BFU-E formation. CFU-GM cluster and colony formation was also unaffected (not shown).

Cross-blocking in the immune rosette test

With red cells sensitized with tetrameric immune complexes prepared of each of the three different Mabs (MB-2D10, LA18.18 or LA-23.40), rosette formation occurred with K562 and HEL cell-line cells. The rosette formation was (nearly) completely blocked when the cells were preincubated with each of the Mabs separately (prediluted 1/10), but not with anti-glycophorin-A Mab. This experiment shows that immune rosetting is specific and indicates that all these Mabs are directed either against the same epitope, or against neighbouring epitopes present on the same red cell membrane structure.

Immunochemical studies

Preliminary studies by Western blot analysis during the 'First International Workshop on Monoclonal Antibodies against human red blood cell and related Antigens' indicated that the MB-2D10 (coded as 27W4), as well as other Rh-related murine Mab, were detecting unidentified diffuse red cell membrane protein bands in the region of 36–44 kDa (Bloy *et al*, 1988b). Further studies were carried out to clarify the specificity of MB-2D10.

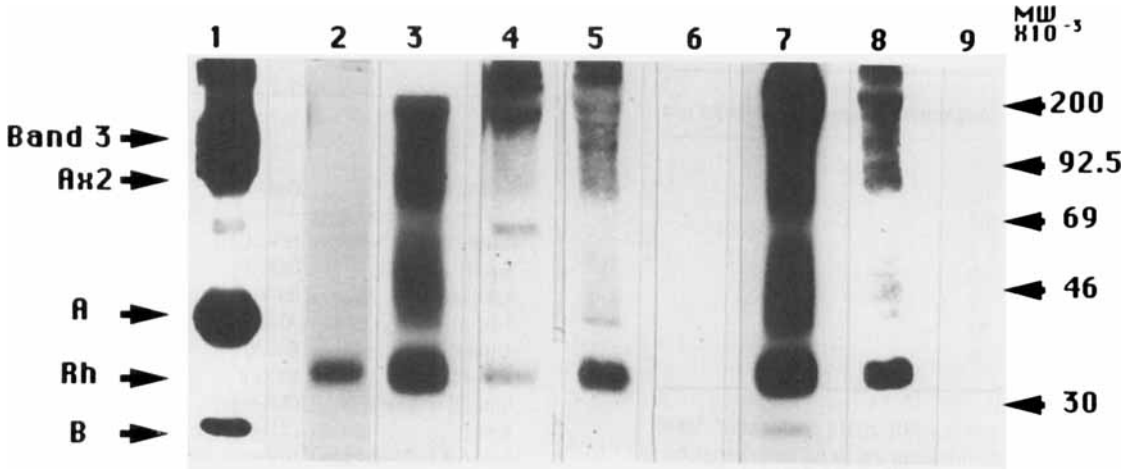

Fig 1. SDS/polyacrylamide gel electrophoresis of red cell membrane proteins immunoprecipitated with MB-2D10. Immunoprecipitates from ^{125}I-labelled cell surface components of cDE/cDE red cells were analysed by SDS/polyacrylamide gel electrophoresis on a 10% separating gel in a discontinuous buffer system. Lane 1: Control profile obtained from ^{125}I-labelled red cell membrane proteins showing the migration position of the main components band 3, glycophorin A monomer and dimer (A and A × 2 respectively), Rh polypeptide (Rh) and glycophorin B (B). Lanes 2 and 6: Immunoprecipitates obtained with human Mab anti-D antibody (H2D5) with intact membranes (lane 2) or membrane lysates (lane 6). Lanes 3–5: Immunoprecipitates obtained with MB-2D10 from intact membranes of native and chymotrypsin-treated cDE/cDE and S-s-U− (Fav.) red cell membranes, respectively. Lanes 7–9: As lanes 3–5, but from membrane lysates.

Table IV. Immunorosette depletion of bone-marrow precursor cells

Mab	Antigen	Immunorosette	CFU-E	BFU-E
MB-2D10	2D10	−	94%	86%
		+	6%	14%
LA-18.18	2D10	−	91%	85%
		+	8%	15%
LA-23.40	2D10	−	94%	80%
		+	6%	20%
MB-CIA2	HLA class II	−	41%	4%
		+	59%	96%
none	−	−	97%	83%
		+	3%	17%

Depletion by rosetting and Ficoll–Isopaque gradient centrifugation of mononuclear bone-marrow cells with tetrameric immune complexes of the Mabs under investigation, anti-glycophorin-A Mab and rat anti-mouse IgG1 Mab. Anti-HLA-class II was used as a positive control. The percentage of colonies, calculated from the total amount of colonies obtained with cells treated in this way, in the ring (−) and pellet fraction (+) (containing rosetted cells) is shown. Results from two other experiments showed identical results.

(A) *Immunoprecipitation.* These studies were done with MB-2D10 and with the human monoclonal anti-D antibody H2D5 as a positive control. The results of the immunoprecipitation studies with ^{125}I-labelled red cell membrane proteins are shown in Fig 1. More material was brought down when the antibody was added to membrane lysates (lane 7) than when added to intact cells (lane 3). A complex pattern of bands was obtained with major components of 30–32 kDa, and diffuse bands at 40–60 kDa and 70–90 kDa (lane 3). In addition, (a) component(s) with a higher molecular mass of more than 100 kDa was present. Under more stringent washing conditions, most bands had a reduced intensity. The 30–32 kDa band, however, remained clearly present under these conditions (not shown). A band migrating at 25 kDa was also found when the Mab was added to membrane lysate (lane 7). This band migrated as glycophorin B as seen from the profile of total ^{125}I-labelled red cell membrane glycoproteins (lane 1). The intensity of the 25 kDa band was variable (not shown). When the red cells were treated with chymotrypsin, before radiolabelling and immunoprecipitation, many bands were no longer detected including the 25 kDa band (data not shown). The 30–32 kDa band, however, as well as the high molecular material (>100 kDa), remained present (lanes 4 and 8).

Studies with Rh-positive S-s-U- red cells showed that no bands could be precipitated with MB-2D10 using membrane lysates (lane 9) whereas the usual complex pattern of bands was obtained with intact membranes (lane 5).

(B) *Determination of the number of antigen sites.* The results of studies with ^{125}I-labelled MB-2D10 and Scatchard plot analysis are shown in Table V. Similar numbers of antigen molecules were measured on red cells of different common Rh phenotype and of the rare Rh phenotype -D-/-D-. Chymotrypsin treatment resulted in a clear reduction in the number of sites, as was already noticed in serological studies described above. Red cells of the phenotype S-s-U- with a glycophorin-B deficiency had increased numbers of sites. On Rh_{null}U negative cells no MB-2D10 molecules were detected. Rh_{null}U positive cells were not tested. An affinity constant of 0·30 to $0{\cdot}7 \times 10^8$ M^{-1} was found with the different red cell types.

(C) *Competitive radio binding assays.* Mab MB-2D10 did not

Table V. Scatchard plot analysis with ^{125}I-MB-2D10 Mab and red cells of different Rh and U phenotypes

Phenotype	No. of molecules per red cell
cDE/cDE	$0{\cdot}49 \times 10^5$
cDE/cDE, chymotrypsin-treated	$0{\cdot}01/0{\cdot}034 \times 10^5$*
cde/cde	$0{\cdot}40 \times 10^5$
-D-/-D-	$0{\cdot}43 \times 10^5$
S-s-U−	$0{\cdot}70 \times 10^5$
Rh_{null} U−	0

Results obtained with the red cells from two donors (experiments performed in triplicate) except for Rh_{null} (one donor).

* The values obtained defined two types of sites, with two different association constants (K_a $0{\cdot}7 \times 10^8$ M^{-1} and $0{\cdot}2 \times 10^8$ M^{-1}). For cDE/cDE and cde/cde K_a was $0{\cdot}7 \times 10^8$ M^{-1}, for -D-/D- and S-s-U− K_a was $0{\cdot}3 \times 10^8$ M^{-1}.

inhibit binding of ^{125}I-labelled human monoclonal anti-D and anti-c to cDE/cDE red cells, but binding of anti-E was significantly inhibited (40%). Such an effect may be due to steric hindrance of anti-E binding by MB-2D10 bound to neighbouring sites. It indicates proximity of both antigens on the red cell membrane. However, the binding of ^{125}I-labelled MB-2D10 was not inhibited by any of the human Mabs (anti-D, anti-c, anti-E).

(D) *Two-dimensional peptide maps.* Peptide maps obtained from the 30–32 kDa polypeptides isolated with MB-2D10 were identical to those obtained with Rh polypeptides isolated with the monoclonal anti-D H2D5 (Blanchard *et al*, 1988) or by chemical purification (Saboori *et al*, 1988) (data not shown). This indicates that the 30–32 kDa band precipitated by MB-2D10 represents true Rh component(s).

DISCUSSION

An antigen, called the 2D10 antigen, described here is a novel antigenic determinant of red cells and also a new marker of erythroid differentiation. It is defined by three murine Mabs: MB-2D10, LA-18.18 and LA-23.40. The 2D10 antigen is expressed on all red cells, except those with the rare Rh_{null}U negative and Rh_{mod}U negative phenotypes, an expression also described for the Duclos-antigen (Rh38) (Habibi *et al*, 1978).

The 2D10 antigen is absent from the erythroid progenitors of the bone marrow (BFU-E and CFU-E). However, it is strongly expressed on immature and mature erythroblastic cells and the erythroblastoid cell lines K562 and HEL, and is apparently expressed in an earlier stage of differentiation than Rh polypeptides, R6A- and LW-glycoproteins and globoside (P-antigen). Therefore, 2D10 antigen may be an interesting marker for erythroid malignancies, such as acute erythroleukaemia (AML-M6) and erythroblastic crisis of CML. Indeed the cells of a limited number of patients with such diseases were found to be positive for the 2D10 marker.

Using normal red cells, complex immunoprecipitation patterns were obtained with MB-2D10, but they always included the 30–32 kDa Rh polypeptides. Moreover, diffuse and overlapping bands from 40–60, 70–90 and larger than 100 kDa were found. In an independent study of Mallison *et al* (1990) on the 2D10 antibodies, the nature of these bands has become clear. The antibodies were found to react with purified preparations of the Rh-related glycoproteins which were called the D50 polypeptide (M_r 45–100 kDa) and the R6A45 polypeptide (M_r 35–52 kDa). These polypeptides are heavily glycosylated, carry ABH-active polysaccharides and coprecipitate with Rh polypeptides and/or the R6A polypeptide in immunoprecipitation studies with human anti-D, anti-c, anti-E antibodies and/or murine monoclonal Rh29 antibodies (R6A, Bric 69) (Moore *et al*, 1982; Gahmberg, 1982; Ridgwell *et al*, 1983; Moore & Green, 1987; Avent *et al*, 1988a, b).

The N-terminal amino acid sequence (first 30 residues) of the D50 and R6A45 polypeptide has been found to be identical (Avent *et al*, 1988b), indicating that they are closely related if not identical polypeptides.

Thus, precipitation of the Rh-related glycoprotein(s) and coprecipitation of Rh-polypeptides accounts for most of the complex band patterns observed by us with MB-2D10.

Concerning the epitope recognized by the 2D10 antibodies, we as well as Mallison *et al* (1990) found it to be resistant to neuraminidase treatment, but sensitive to a number of proteases, notably bromelin, pronase and chymotrypsin. Moreover Mallison *et al* found the epitope to be sensitive to endoglycosidase F treatment, indicating that it is carbohydrate (N-glycan) dependent.

Serological and immunochemical studies on the blood of individuals with the rare Rh_{null} phenotype have shown that multiple defects exist in their red cells (for review see Issitt, 1985; Nash & Shojania, 1987; Anstee & Tanner, 1988). Such individuals have chronic haemolysis with stomatocytosis and spherocytosis, the so-called Rh deficiency syndrome. Apart from an abnormal shape the red cells have an increased permeability towards cations and a disturbed membrane phospholipid asymmetry. The surface membrane of these cells lacks all antigens of the Rh- and LW-blood group system due to absence of Rh polypeptides and LW-glycoproteins. Moreover, the Rh polypeptide related structure identified by the murine monoclonal antibodies R6A and Bric 65 is absent, and that identified by CD47 antibodies (Bric 125, Bric 126) is severely deficient (Ridgwell *et al*, 1983; Mallison *et al*, 1990; Avent *et al*, 1988a). The absence or deficiency of so many different red cell surface membrane structures in the Rh_{null} phenotype as well as the complex immunoprecipitation patterns observed with normal red cells and different Rh- and Rh-related antibodies, as discussed above, indicate that these structures associate in the normal membrane as a functional complex, the Rh protein complex (Cartron, 1987; Avent *et al*, 1988a). The red cell abnormalities in Rh_{null} individuals show that such a complex is necessary for normal membrane integrity and indicate that the deficient production of one component may lead to a disturbed assembly of the whole complex.

Although the molecular defects responsible for Rh deficiency phenotypes are not yet known, genetic studies have

indicated that at least three different types may exist: the amorph Rh_{null} type, the regulator Rh_{null} type and a modified type called Rh_{mod} (Issitt, 1985). All appear to be caused by homozygosity of rare recessive genes. The regulator type of Rh_{null} (18 examples are recorded), is much more common that the amorph type (only three examples listed) (published in the literature, and MRC Blood Group Unit, unpublished observations).

In the amorph type (a) silent gene(s), $- - -$, appears to be present at the Rh locus. Thus, the deficient components are probably the Rh polypeptides and also the $R6A_{32}$ polypeptide, a closely related but not identical structure (Avent *et al*, 1988b). Two of the three amorph type Rh_{null} individuals were tested; both were 2D10 positive, both were U+.

In the regulator type a suppressor gene ($X^{o}r$) is postulated to be present at a locus independent of Rh. It is possible that in this case a gene is defective which codes for one of the other components of the Rh protein complex necessary for its normal assembly. In fact in those Rh_{null} individuals who were found to be 2D10 negative it can be speculated that the primary defect may exist in the gene coding for the 2D10 polypeptide. Of the five regulator type Rh_{null} individuals tested, four (all U−) were 2D10 negative and only one, a U+, was positive.

In the modified type of Rh deficiency phenotype, Rh_{mod}, the existence is postulated on an allele X^{q} which depresses but does not completely abolish expression of Rh antigens. This phenotype is thought to be heterozygous since there is a wide variation in the strength of Rh antigens observed in different Rh_{mod} propositi. Of the two Rh_{mod} samples tested one had normal 2D10 expression and the other was 2D10 negative.

Apart from the abnormalities in Rh_{null} and Rh_{mod} discussed above there also exists a qualitative abnormality in glycophorin B (GPB) (Issitt, 1985; Dahr *et al*, 1987). In all forms of Rh_{null} and Rh_{mod} studied so far the amount of GPB on the red cell surface membrane is reduced to about 30%. There is a concomitant (slight) weakening in the expression of different GPB associated alloantigens (S, s, 'N'). Moreover, there is a strong depression of the U-antigen and the U-like antigen Duclos (Rh38) in most regulator type Rh_{null} cases, but not in amorph types (except for members of a Norwegian family of the amorph type (Race & Sanger, 1975)).

Evidence has been forwarded that the Duclos- and the U-antigen are located in residues 33–40 of GPB situated near the cell surface membrane (Dahr & Moulds, 1987). Although in our studies on Rh_{null} red cells 2D10 antigen expression was always associated with Duclos- and U-antigen expression, they are clearly distinct antigens. GPB deficient (S-s-U-) red cells reacted normally with 2D10 antibodies, as did the red cells of Mrs Duclos, recovered from many years of storage in liquid nitrogen (Le Pennec *et al*, 1988). Thus, the most likely explanation for the serological association of 2D10, U− and Duclos-antigens on different Rh_{null} cells is that the latter two antigens require the association of GPB and 2D10 glycoprotein for optimal activity.

That such an interaction indeed occurs was indicated by our immunoprecipitation experiments. Notably, in experiments on membrane lysates of normal cells a band of 25 kDa probably equivalent to GPB coprecipitated. This was not found with intact membranes, but this method was less sensitive and less material was brought down.

An unexplained finding is that we were not able to precipitate 2D10 material from membrane lysates of GPB deficient (S-s-U-) red cells, while with intact membranes of such cells the usual complex pattern was found. Possibly, GPB is necessary for the correct expression of the 2D10 epitope in detergent micelles obtained after membrane solubilization in Triton X-100.

In conclusion, the combined serological and immunochemical data presented here, and recently reported by Mallison *et al* (1989), indicate that the 2D10 antigen detected by our Mabs, is not located on the Rh polypeptides, the LW^{ab} protein or glycophorin B, but is carried on distinct membrane proteins, which are probably the so-called Rh-related glycoproteins. The antibodies will be useful to study these Rh-related glycoproteins in more detail, to unravel the association with other components of the Rh protein cluster and their role in maintaining the integrity of the normal red cell membrane. They are useful tools for the analysis of red cells of individuals with the Rh deficiency syndrome, for studying red cell differentiation and maturation, and in leukaemia typing, notably to detect involvement of the red cell lineage.

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