Report

# The production, serologic evaluation, and epitope mapping of ten murine monoclonal Dombrock antibodies

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The Dombrock (Do) glycoprotein is a glycosylphosphatidylinositol (GPI)-linked membrane protein carrying Dombrock blood group antigens. There are no standardized typing reagents for Do$^a$ or Do$^b$. We have developed ten different monoclonal antibodies (MoAbs) that are specific for Dombrock. The objectives of this study were to characterize these MoAbs serologically and determine the epitopes they recognize. MoAbs were generated by standard fusion methods. Mice were immunized with transfected human embryonic kidney 293T cells expressing high levels of Do$^a$ or Do$^b$. The MoAbs were tested serologically with untreated and enzymatically or chemically modified red blood cells (RBCs). Serologic inhibition studies were performed with synthetic peptides corresponding to Do$^a$ and Do$^b$ amino acid sequences. Pepscan epitope analysis was done on an array of immobilized tridecapeptides corresponding to the full-length polypeptide. All ten antibodies were serologically specific for Dombrock. Eight of the antibodies recognized epitopes that were resistant to treatment with ficin, pronase, α-chymotrypsin, and neuraminidase, but sensitive to trypsin and 0.2 M dithiothreitol (DTT). Five have anti-Do$^b$-like specificity. The epitope recognized by MIMA-52 was neuraminidase sensitive, and MIMA-127 epitope recognized a DTT-resistant, linear epitope $^{90}$QKNYFRMWQK$^{99}$ of the Dombrock polypeptide. MIMA-127 was the only one of the ten Dombrock MoAbs mapped to a specific sequence of the Dombrock glycoprotein; the other nine MoAbs did not provide a specific peptide binding pattern. The other MoAbs could not be mapped as they most likely recognize nonlinear, conformation-dependent epitopes, as is evident by their sensitivity to reduction of disulfide bonds by DTT. The dependence of some epitopes on antigen glycosylation is also a possibility. ***Immunohematology* 2012;28:124–9.**

**Key Words:** Dombrock, hemagglutination, monoclonal antibodies, Pepscan analysis, synthetic peptides

The Dombrock (Do) blood group glycoprotein (CD297) is an interesting glycosylphosphatidylinositol (GPI)-linked component of human erythrocyte membranes, identified as a polymorphic member of the adenosine diphosphate (ADP)-ribosyltransferase gene family.[1,2] The Dombrock glycoprotein carries the polymorphic Do$^a$ and Do$^b$ antigens and other high-prevalence antigens: Gregory (Gy$^a$), Holley (Hy), Joseph (Jo$^a$), DOYA, DOMR, and DOLG.[3–7] Rare human antibodies to the Dombrock antigens, although only weakly reactive in vitro, have caused acute and delayed hemolytic transfusion reactions.[8–10] The poor reactivity of the antibodies and the lack of fully characterized antisera and commercial red blood cells (RBCs) have created problems for resolving serologically difficult patient samples. Considering the importance of using well-defined antibodies in the studies on the Dombrock glycoprotein, we have produced several novel murine monoclonal antibodies (MoAbs) by immunizing mice with human embryonic kidney (HEK) cells that were transiently transfected to express the Dombrock antigens Do$^a$ or Do$^b$. These antibodies were evaluated for their serologic specificity and were subjected to Pepscan epitope mapping and to peptide inhibition studies.

## Materials and Methods

### Transfection and Expression of Dombrock in HEK 293T Cells

The full-length cDNA coding for the Do$^a$ and Do$^b$ transcripts was cloned in pIRES2-EGFP (pDI2E) vector (Clontech, Mountain View, CA), allowing the simultaneous expression of green fluorescent protein (GFP) and the Dombrock gene.[2] HEK 293T cells were transiently transfected with 10 μg of Do$^a$ and Do$^b$ cDNA expression constructs by the calcium phosphate transfection method and analyzed for surface expression using anti-Gy$^a$ by flow cytometry as described previously.[11] Briefly, $10^6$ transfected HEK 293T cells were incubated for 30 minutes at 37°C with anti-Gy$^a$ and, after several washes, were stained with phycoerythrin (PE)-conjugated horse anti-mouse IgG (H+L) (Vector Laboratories, Burlingame, CA), washed, and analyzed by flow cytometry (Canto, Becton Dickinson, San Jose, CA). On average, Dombrock expression was detected by flow cytometry on approximately 50 percent of transfected cells, consistent with a transfection efficiency of 50 percent (data not shown).

### Hybridoma Production

HEK cells transiently transfected with Dombrock cDNA corresponding to Do$^a$ or Do$^b$ were used to immunize BALB/c

mice three times during a 6-week period by intravenous (IV) injection of $2 \times 10^6$ tHEK cells plus a murine CpG adjuvant.[12] Once adequate immunization was achieved, as demonstrated by hemagglutination of antigen-positive human RBCs, polyethylene glycol (PEG; Sigma Chemical Company, St. Louis, MO) fusions were done between the mouse splenocytes and X63-Ag8.653 mouse myeloma fusion partner cells. Briefly, the mouse splenocytes were mixed with the fusion partner X63.Ag8.653 cells at a ratio of roughly 3:1 and washed with phosphate-buffered saline (PBS) without calcium ($Ca^{2+}$) or magnesium ($Mg^{2+}$) (GIBCO/Invitrogen, Grand Island, NY). Fusions were achieved by the addition of 1 mL of PEG (with 5% DMSO; Sigma) dropwise slowly for 60 seconds with constant mixing followed by constant mixing at 37°C for 90 seconds. Then 1 mL of PBS (without $Ca^{2+}$ and $Mg^{2+}$) was added in a dropwise fashion for 60 seconds, repeated once, and then 1 mL of PBS was added for 30 seconds, repeated once, and then topped off to a volume of 15 mL with PBS. The cells were then allowed to rest at 37°C, followed by gentle centrifugation at 1000 rpm for 5 minutes and washed in warmed HAMS/DF-12 culture medium (Sigma) with 10 percent fetal bovine serum added (Hyclone Laboratories, Inc., Logan, UT). The cells were then plated onto sterile 96-well tissue culture trays (BD Falcon, Franklin Lakes, NJ) at 1.5 million cells per well in HAMS/DF-12 with hypoxanthine, aminopterin, and thymidine added (HAT; Sigma). After 7 days, 100 µL of additional culture medium with supplemental growth factors (StemCell Technologies, Vancouver, BC) was added to each well.

### Screening Fusion Trays for Antibody Secretion

The plates were analyzed 14 days after fusion for the presence of antibodies by hemagglutination as follows: a 50-µL aliquot of supernatant fluid from each well was added to the corresponding wells of a 96-well v-bottom assay tray (Greiner Bio-One, Longwood, FL), 25 µL of a 1 percent RBC suspension of screening cells was added, and the trays were mixed on a titer plate shaker (Lab-Line Instruments, Melrose Park, IL). After 30 minutes the trays were observed for direct agglutination. The wells were washed by adding 100 µL PBS, mixing, and centrifuging at 1000 rpm for 30 seconds. The wash PBS was flicked off by inverting the tray over the sink and blotting dry on a paper towel. For indirect agglutination, 50 µL of anti-mouse IgG (The Binding Site, San Diego, CA) that had been diluted 1:100 in 6 percent albumin in PBS was added to each well, mixed, and incubated for 30 minutes, then observed for agglutination. The desired hybrids were then cloned three times by limiting dilution to assure monoclonality.

### Serologic Evaluation and Synthetic Peptide Inhibition Studies

The final culture of each hybrid was grown in vitro, and the supernatant fluid was tested for reactivity by standard tube and gel hemagglutination methods using normal and chemically or enzymatically treated RBCs. Enzyme treatments with papain, trypsin, α-chymotrypsin, pronase, and neuraminidase and chemical treatment with 0.2 M DTT were done using standard methods described elsewhere.[13] Hemagglutination inhibitions were performed in two stages: first incubating equal volumes (50 µL) of antibody with the synthetic peptide at a concentration of 1 µg/µL, mixing and incubating 30 minutes. Next, one drop of 3 percent RBCs suspended in PBS was added to each tube and hemagglutination was performed as described earlier to complete each test.

### Epitope Mapping Using Immobilized Peptides

Eighty-one tridecapeptides (peptides consisting of 13 amino acids) synthesized on pins, which covered the sequence corresponding to the full-length membrane-bound Dombrock protein (i.e., amino acid residues 45–297, which lacks the leader sequence and phosphatidylinositol glycan-anchoring sequences), were obtained from Mimotopes (Clayton, Victoria, Australia). The selected decapeptides were synthesized using the epitope scanning kit purchased from Mimotopes. The decapeptides were synthesized on activated plastic pins fixed to the plate corresponding to a 96-well microtiter plate. The syntheses were done by stepwise elongation of the peptides from *C*- to *N*-terminus, following the manufacturer's instructions.

Binding of the antibodies to tridecapeptides and decapeptides on pins was tested as described.[14–17] The pins were consecutively immersed (with washing between the incubations) in the wells containing (1) the test MoAbs, incubated overnight at 4°C, (2) alkaline phosphatase–conjugated goat anti-mouse IgG (Dako, Copenhagen, Denmark), incubated for 1 hour at ambient room temperature, and (3) phosphatase substrate tablets (Sigma). The absorbance of the substrate solution samples was read at 405 nm in a microtiter plate reader (PerkinElmer 2300 multiplate reader, Melrose Park, IL). The test antibodies were used at dilutions of 1:20, if not stated otherwise.

## Results

### Serologic Characterization of the Antibodies

The MoAbs from ten hybridomas were Dombrock-specific as they reacted with all Dombrock-positive RBCs

**Table 1.** Serologic reactivity of Dombrock monoclonal antibodies

| Monoclonal antibody | Clone ID | Specificity | Ficin | Papain | Trypsin | Chymotrypsin | Neuraminidase | DTT |
|---|---|---|---|---|---|---|---|---|
| MIMA-52 | 10B5 | Do protein | NT | R | S | R | **S** | **W** |
| MIMA-53 | 8E9 | Do protein | NT | R | S | R | R | S |
| MIMA-55 | 7H6/F7 | $Do^b$ | NT | R | S | R | R | S |
| MIMA-64 | 7H6/B3 | $Do^b$ | NT | R | S | **W** | R | S |
| MIMA-73 | 15D6 | $Do^b$ | NT | R | S | R | R | S |
| MIMA-98 | 7D10 | Do protein | R | NT | S | **W** | R | S |
| MIMA-123 | 6B5 | $Do^b$ | R | NT | S | R | R | S |
| MIMA-125 | 5A11 | Do protein | NT | R | S | R | R | S |
| MIMA-127 | 7C8 | Do protein | R | NT | S | **S** | NT | **R** |
| MIMA-143 | 1G8 | $Do^b$ | NT | R | S | R | R | S |

ID= identification; DTT = dithiothreitol; NT = not tested; R = resistant; S = sensitive; W = weaker reactivity after treatment.
Bold font highlights the significant differences in MoAb reactivity.

but not with Gy(a–) and Hy– RBCs. Five were anti-$Do^b$-like by hemagglutination as they reacted, after selected dilution in 6 percent albumin in PBS, with Do(a–b+) or Do(a+b+) RBCs, but not with several examples of Do(a+b–) RBCs. The remaining five recognized epitopes independent of the $Do^a/Do^b$ polymorphism. The MoAbs, all isotype IgG2a, had similar reactivity patterns with enzymatically treated or chemically modified RBCs, with a few exceptions (Table 1). The epitopes recognized by all or most MoAbs were resistant to ficin or pronase, α-chymotrypsin, and neuraminidase treatment and were sensitive to trypsin and 0.2 M DTT treatment. The most important exceptions were MIMA-52, which was unique in that it was specific for a neuraminidase-sensitive epitope, and MIMA-127, which recognized an epitope sensitive to α-chymotrypsin and resistant to DTT treatment.

### Pepscan Epitope Mapping

The results of hemagglutination of protease-treated RBCs did not reveal any information about the epitopes that each MoAb recognized on the Dombrock molecule because it is not known which peptide bonds of the Dombrock polypeptide chain are hydrolyzed by the proteases used. For initial epitope mapping a panel of 81 tridecapeptides, covering the entire membrane-bound sequence of the Dombrock glycoprotein, was used (Fig. 1). Each tridecapeptide differed by three

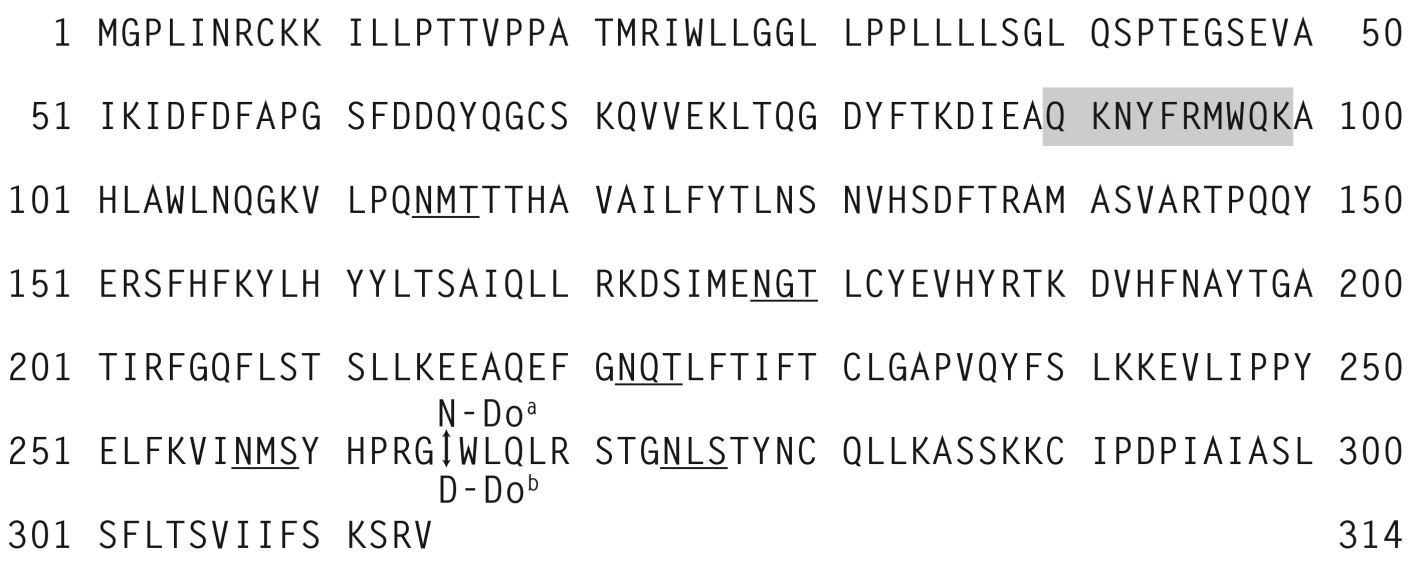


**Fig. 1** Amino acid sequence of the Dombrock glycoprotein. The blood group $Do^a/Do^b$ polymorphism at amino acid residue 265 is shown. Five potential *N*-glycosylation sites are underlined. Epitope for the MIMA-127 antibody is highlighted in gray.

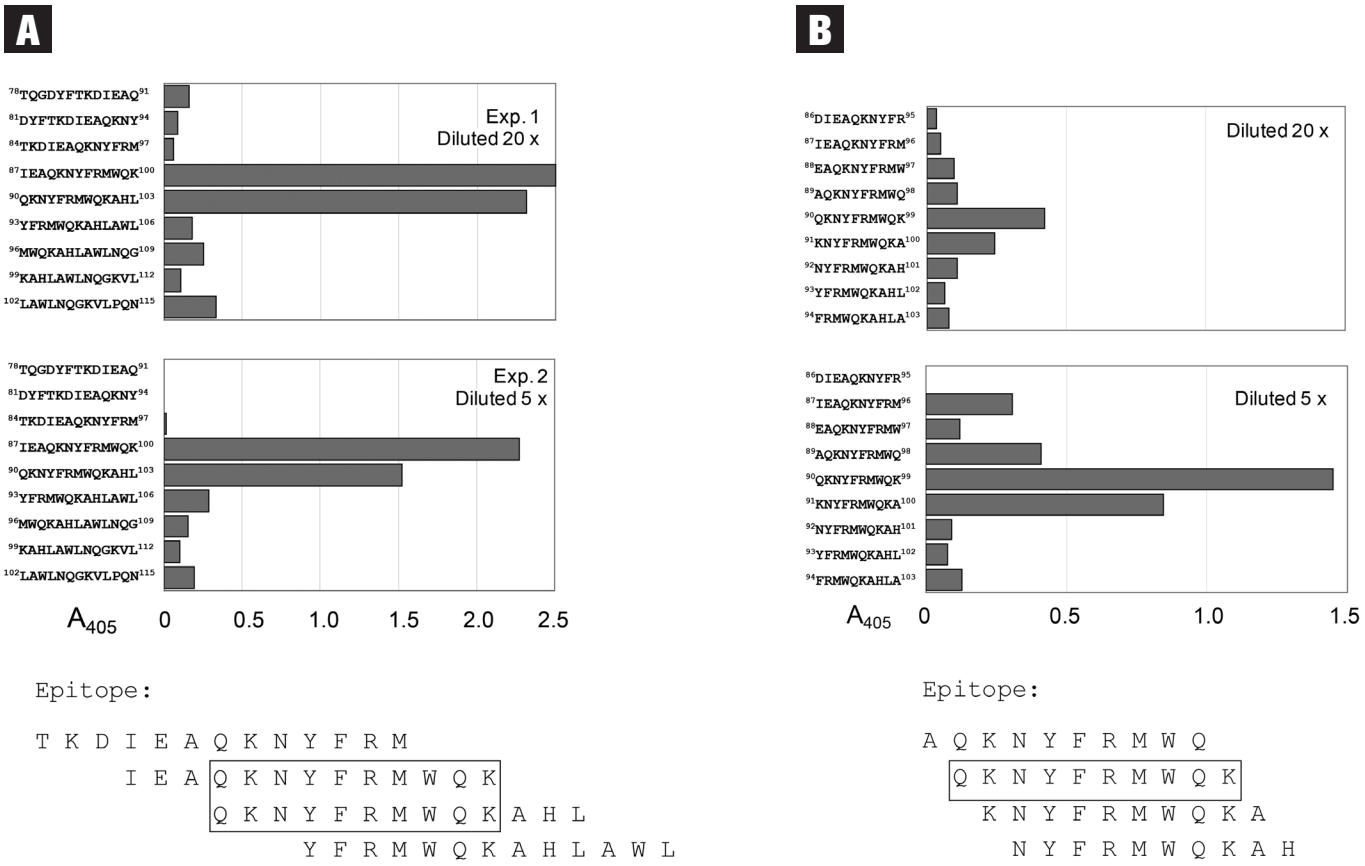


**Fig. 2** Mapping the MIMA-127. Pins made with the peptides listed were incubated with MIMA-127 followed by mouse IgG, then alkaline phosphatase, and the absorbance was measured at 405 nm. Binding to the tridecapeptides (**A**) and decapeptides (**B**) is shown. Dilution of the antibody solution used in the experiments is indicated. The sequence of the most active tridecapeptides and a decapeptide is shown and their common sequence fragment (epitope) is boxed.

amino acids sequentially from the next and corresponded to amino acid residues 45–297 of the Dombrock glycoprotein (UniProt entry Q93070, Fig. 1). Of these ten MoAbs, only one antibody, MIMA-127, displayed a specific and strong binding to the peptides 87–100 (IEAQKNYFRMWQK) and 90–103 (QKNYFRMWQKAHL; Fig. 2A). To verify this observation, a set of nine decapeptides (shown in Fig. 2B), corresponding to the sequence of amino acids $^{86}$DIEAQKNYFRMWQKAHLA$^{103}$ of Dombrock, was synthesized on pins. MIMA-127 bound most strongly to the decapeptide 90–99 and more weakly to the peptide 91–100 (Fig. 2B). The binding to the decapeptides was weaker than to the respective tridecapeptides (at 20-fold antibody dilution) and was increased when the more concentrated dilution of the antibody was used. Thus, our results confirmed that MIMA-127 recognizes the sequence $^{90}$QKNYFRMWQK$^{99}$, present in all of the most active peptides. The stronger reactivity of the antibody with the tridecapeptides than with decapeptides suggests that amino acid residues flanking this epitope may play a role in antibody binding owing to conformational changes in the protein. Testing other antibodies at fivefold dilution did not increase their binding to any peptides.

The Do$^a$ and Do$^b$ blood group antigens differ by the presence of asparagine (Asn) and aspartic acid (Asp), respectively, at position 265 of the Dombrock glycoprotein. Our set of tridecapeptides contained Asn at this position, which contributed to a lack of binding of antibodies identified serologically as anti-Do$^b$-like. To test these antibodies, two sets of six decapeptides, covering the amino acid sequence 258–272 of Do$^b$ and differing by the presence of Asn265 or Asp265, were used. However, no Do$^b$-like antibodies bound to these peptides (data not shown).

### Inhibition of Antibodies With Soluble Peptides

Synthetic peptides corresponding to the amino acid sequence surrounding the Do$^a$/Do$^b$ polymorphism at position 265 were used in inhibition assays. We used Do$^b$-specific peptides that were either 15 ($^{261}$HPRGDWLQLRSTGNL$^{275}$),

16 ($^{255}$VINMSYHPRGDWLQLR$^{270}$), or 21 ($^{255}$VINMSYHPRG DWLQLRSTGNL$^{275}$) amino acids in length. None of the Dombrock MoAbs were inhibited by these synthetic peptides.

## Discussion

We have produced ten different murine hybridoma cell lines that secreted monoclonal antibodies specific to the Dombrock glycoprotein, and five of them were anti-Do$^b$-like by serologic testing. An attempt to identify the epitopes detected by the MoAbs on the Dombrock glycoprotein was successful only for MIMA-127. Our results showed that MIMA-127 recognizes a relatively long linear epitope formed by amino acid residues 90–99 of the Dombrock glycoprotein (Fig. 2). The results indicated that peptide 89–98, which did not contain Lys99, and peptide 91–100 without Gln90, were less active than peptide 90–99. Lack of specific reactivity of other antibodies with synthetic peptides suggests that the MoAbs recognize conformation-dependent nonlinear epitopes. The Dombrock glycoprotein contains five cysteine residues that can form two disulfide bonds, resulting in the formation of nonlinear epitopes.[2] This conclusion is strongly supported by our finding that all epitopes recognized by the MoAbs were destroyed by treatment of RBCs with DTT (reduction of disulfide bonds), except the epitope for MIMA-127, which was DTT-resistant.

On the other hand, the lack of reactivity of anti-Do$^b$-like MoAbs with immobilized and soluble peptides covering the blood group polymorphism region may be attributable to the absence of glycosylation on the synthetic peptides. There are two potential *N*-glycosylation sites, at Asn257 and Asn274 residues flanking the Do$^a$/Do$^b$ (Asn265Asp) polymorphism site. The effect of glycosylation on peptidic epitope reactivity can differ: an antibody can recognize both amino acids and carbohydrates (glycopeptidic epitope), or an oligosaccharide chain may interact with amino acid residues in a manner that changes the local conformation of the polypeptide chain and may result in exposing or burying the adjacent peptidic epitope.[18] One of these possibilities can also apply to the reactivity of MIMA-52, which recognized a sialic acid–dependent epitope.

A lack of interaction of other antibodies either with immobilized or with soluble synthetic linear peptides and sensitivity of the epitopes to DTT suggests that most Dombrock monoclonal antibodies recognize nonlinear, conformation-dependent epitopes. For some of the antibodies the effect of antigen glycosylation on the reactivity of epitopes cannot be ruled out. However, all antibodies obtained were shown to be specific for the Dombrock protein and can be used for its identification on the erythrocyte surface.

Despite several attempts using similar methods of immunization with transfected cells, we were not successful in producing a monoclonal antibody with Do$^a$-like specificity. It may be worth attempting immunization with synthetic peptides that are specific for Do$^a$; however, conformation and glycosylation appear to play important roles in antigen recognition to stimulate the immune system for antibodies with Do$^a$ specificity.

Antibodies to Dombrock blood group antigens are typically weakly reactive, and thus they are often misidentified. The monoclonal antibodies reported in this manuscript are typical in that they are not strongly reactive, and their use in serologic testing must be carefully controlled. Testing in gel cards with anti-mouse IgG has proved to be the best method for antigen typing to identify antigen-negative donor units and to be helpful in resolving serologically difficult patient samples by phenotyping their RBCs.[19] Though the mouse IgG gel cards are currently not available in the United States, we developed a method of converting neutral gel cards into mouse IgG gel cards for this purpose.

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