Close Association of the First and Fourth Extracellular Domains of the Duffy Antigen/Receptor for Chemokines by a Disulfide Bond Is Required for Ligand Binding*

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It has been demonstrated that the promiscuous chemokine binding profile of the Duffy antigen/receptor for chemokines (DARC) is given by its extracellular NH2-terminal region. However, the relationship among the Fy6, Fy-a/b, and Fy3 epitopes, localized in the first and fourth extracellular domains of DARC, respectively, and the chemokine binding sites remained a matter of controversy. Here, we performed cross-displacement and cross-inhibition experiments indicating that all anti-Fy6, anti-Fy-a, and anti-Fy3 monoclonal antibodies and inter leukin 8 are antagonists for binding to red cells. Biopanning of phage peptide libraries with an anti-Fy6 monoclonal antibody led to the identification of the motif Phe22-Glu23, the mutation of which altered the binding of both anti-Fy6 and chemokines (interleukin 8, MGSa, RANTES (regulated on activation normal T cell expressed)) to DARC transfectants. These results characterized the core of the Fy6 epitope and provided definitive proof of the tight relationship between Fy6 and the chemokine receptor site. Analysis of red cells treated by sulphydryl group-modifying reagents suggested that the chemokine receptor function of DARC required the integrity of disulfide bond(s) but not that of free sulphydryl group(s). Accordingly, mutation of cysteines 51 and 276 abolished chemokine binding to DARC transfec tants. Altogether, our results suggested that the chemokine binding pocket of DARC included sequences located in the first and fourth extracellular domains which are brought into close vicinity by a disulfide bridge.

The Duffy blood group antigens were first recognized as the erythrocyte receptor for malaria parasites and for chemokines because Duffy-positive but not Duffy-negative erythrocytes can be invaded by Plasmodium vivax (1) and Plasmodium knowlesi (2) and can bind interleukin (IL)-8 (3, 4). The major subunit of the Duffy antigens, glycoprotein gpD (36–46 kDa) (5), exhibits significant protein sequence homology with the human and rabbit IL-8 receptors (6) and is most likely organized into seven transmembrane domains like all members of the G-protein-coupled chemokine receptors (7). Analysis of human cell line transfec tants indicated that gpD is sufficient for Fy3, Fy6 (7, 8), and Fy-a/b (9) blood group antigen expression, as well as for the binding of chemokines of both the CC (RANTES, MCP-1) and CXC (IL-8, MGSa) classes (7, 8). Thus, gpD is now referred as the promiscuous chemokine receptor (8) or as the Duffy antigen/receptor for chemokines (DARC) (10). Since chemokines constitute a family of proinflammatory cytokines that mainly activate leukocytes and cause cell recruitment, it has been postulated that the red cell promiscuous chemokine receptor could act only as a sink or scavenger to bind and inactivate chemokines released into circulation (3). In Duffy-negative black individuals (Fy(a-b)-), the DARC gene is not expressed in erythroid cells (6) because of a mutation in a binding site for the h-GATA-1 erythroid-specific transcription factor (11, 12). However, it is normally active in non-erythroid tissues, which should explain that Duffy-negative donors are apparently healthy (13). Previous immunohistochemical studies indicated that the non-erythroid expression of DARC is restricted to the endothelial cells lining post-capillary venules and sinusoids, at least in kidney, breast, and spleen, respectively (14, 15), but present studies showed that DARC is also present in the epithelial cells of the kidney collecting ducts and lung alveoli (16). Furthermore, cDNA sequence analysis indicated that the renal and placental chemokine receptor is identical to the erythroid isoform (7, 11, 14, 16). Chemokines bound to cell line transfec tants expressing this DARC isoform are internalized rapidly (15), but there was no evidence for signal transduction through the recombinant DARC challenged by various chemokines (7). Thus, the function of the Duffy antigen as a chemokine receptor expressed on endothelial cells of post-capillary venules, an important site for chemokine-induced leukocyte trafficking, remains to be determined. Expression of the Fy6 antigen and chemokine binding to Purkinje cells in the cerebellum (17) also raised the question of the biological role of DARC in brain, since the different size of the DARC-related mRNA in brain compared with other tissues, 8.5 versus 1.35 kilobases (6), suggests that a specific DARC isoform with a peculiar function might be expressed in this tissue.

Even though the precise function of DARC in various cell types and tissues is not yet understood, its receptor properties for ligands of so great a biological significance as malaria parasites and chemokines have prompted several groups to perform receptor/ligand interaction analysis. The binding site for the P. vivax and P. knowlesi ligands (the cysteine-rich domain or region II) has been mapped between amino acids 9

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§ The abbreviations used are: IL, interleukin; RANTES, regulated on activation normal T cell expressed; MCP, monocyte chemotactic protein; MGSa, melanoma growth-stimulating activity; DARC, Duffy antigen/receptor for chemokines; mAb, monoclonal antibody; NEM, N-ethylmaleimide; DTT, dithiothreitol; RhD, Rhesus D.
and 42 of DARC (18). Analysis of DARC/IL-8RB chimeric receptors localized the promiscuous chemokine binding profile of DARC to the NH₂-terminal extracellular domain (19). Preincubation of red cells or DARC transfectant cells with anti-Fy6 mAb was shown to inhibit subsequent binding of chemokines, establishing an association between the Fy6 epitope, localized on the NH₂-terminal domain, and the chemokine binding site (4, 8, 19). However, one report suggested that the binding sites for anti-Fy6 and for chemokines might be distinct, since in a converse experiment preincubation of red cells with IL-8 failed to interfere with Duffy antigen expression (20). Furthermore, contradicting results regarding the participation or nonparticipation of the Fy3 epitope (localized on the third extracellular loop) to the chemokine binding site have been proposed in two recent reports (18, 21).

To clarify these issues, we performed a detailed analysis of the receptor/ligand interaction between DARC and chemokines by using three anti-Fy mAbs in the same set of competition experiments and by analyzing the effect of mutations in the core of the Fy6 epitope, mapped by biopanning of a phage display peptide library. By inference with the model of the IL-8 receptors (22, 23), we also analyzed the role of extracellularly exposed cysteine residues in the chemokine binding properties of DARC, through their potential participation to form reactive sulfhydryl groups and/or to a disulfide bridge that brings the first and fourth extracellular domains of DARC into close proximity.

EXPERIMENTAL PROCEDURES

Materials—125I-IL-8, 125I-RANTES, and 125I-MGSA (specific activity, 2,200 Ci/mmol) were obtained from DuPont NEN. Unlabeled recombinant IL-8 was prepared as described previously (24). Unlabeled RANTES and MGSA were from R&D Systems (Abingdon, United Kingdom). The anti-Fy6 (13A) mAb was described elsewhere (25). The anti-Fy3 (CRC-512) and anti-Fya mAbs were kindly provided by Dr. Makato Uchikawa (Japanese Red Cross, Tokyo, Japan) and Dr. F. Buffiere (ETS, Bordeaux, France), respectively. Anti-Fy* and anti-Fy human polyclonal antibodies were from Ortho Diagnostic Systems (Raritan, NJ).

Erythrocyte Samples—Whole blood from healthy donors was collected on EDTA, and the Fy phenotypes were determined by agglutination. Polyvalent antibodies were from Ortho Diagnostic Systems (Raritan, NJ). Erythrocytes were isolated by standard techniques. Modified on EDTA, and the Fy phenotypes were determined by agglutination. Polyvalent antibodies were from Ortho Diagnostic Systems (Raritan, NJ).

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Phage Display Libraries—6-residue and 15-residue phage display libraries were generously provided by Dr. George P. Smith (University of Missouri, Columbia) and screened as recommended (28) with the biotinylated anti-Fya mAb. After three rounds of biopanning, 60 positive phage clones were selected from the two libraries and characterized by insert sequencing.

RESULTS AND DISCUSSION

Cross-displacement Binding of IL-8 and Anti-Fy mAbs to Human Red Cells—Displacement experiments were performed by preincubation of Fy(a+b–) red cells with 125I-IL-8 followed by the addition of various concentrations of anti-Fya*, anti-Fy6, and anti-Fy3 mAbs. The amount of remaining radiolabeled ligand bound to red cells was determined as described under “Experimental Procedures.” As shown in Fig. 1A, 125I-IL-8 could be removed from its binding site by all three anti-Fy mAbs in a dose-dependent fashion, but not by a control mAb specific for RhD. However, IL-8 was more efficiently displaced by anti-Fy6 than by anti-Fya* or anti-Fy3, since when mAbs were used at half their saturating concentration, displacements of 80% and 25% were observed with anti-Fy6 and anti-Fya* or anti-Fy3, respectively.

In a converse experiment, the red cells were preincubated with saturating concentrations of the anti-Fy mAbs prior to the addition of increasing amounts of cold IL-8. The cells were then incubated with a fluorescein isothiocyanate-conjugated second antibody and analyzed by flow cytometry. Fig. 1B shows that, in a dose-dependent fashion, IL-8 could displace the three bound anti-Fy mAbs and that a plateau corresponding to 30% and 40% displacement was reached when 200 ng IL-8 was added after incubation with anti-Fy6 and anti-Fya* or anti-Fy3, respectively.

Cross-inhibition Binding of IL-8 and Anti-Fy mAbs to Human Red Cells—Inhibition analyses were performed by incubating Fy(a+b–) red blood cells with increasing amounts of anti-Fy or control anti-RhD mAbs prior to the addition of 0.5 nM 125I-IL-8. As shown in Fig. 2A, all anti-Fy mAbs dose-responsively inhibited the binding of 125I-IL-8, whereas the control anti-RhD did not. A 90–100% inhibition was obtained with all the use of polymerase chain reaction primers carrying appropriate nucleotide substitutions and the Quick Change site-directed mutagenesis Kit (Stratagene). Inserts of the mutated plasmids were sequenced as above.

Flow Cytometry Analysis—Expression of Duffy antigens on red cells or transfectant cell lines was measured on a FACSScan flow cytometer (Becton Dickinson, San Jose, CA) using anti-Fy6, anti-Fya, and anti-Fy3 mAbs. 3 × 10⁵ cells were incubated for 60 min at 22 °C with appropriate dilution of antibody in 0.15 M phosphate-buffered saline. After washing with phosphate-buffered saline supplemented with 0.5% bovine serum albumin, the cell suspension was incubated with fluorochrome-conjugated anti-human IgG (H+L) (Immunotech, Marseille, France). After another washing step, 0.1 ng of propidium iodide was finally added to 1 ml of cell suspension. Propidium iodide-positive cells (dead cells) were excluded from analysis. Fy(a–b–) red cells and irrelevant mouse and human mAbs were used as negative control. For inhibition and displacement experiments, red cells were incubated with anti-Fy mAbs prior to or after incubation with various concentrations of unlabeled IL-8 at 4 °C for 1 h.

Receptor Binding Assays—COS-7 and K562 cell expressing recombinant wild-type and mutant DARC and red cells were analyzed (tripli-cate) for the ability to bind 125I-IL-8, 125I-RANTES, and 125I-MGSA. 10⁶ red cells and 10⁵ transfected cells were incubated with 0.5 nM radiola-beled chemokines in the presence or absence of 200 nM unlabeled chemokines. Cells were incubated at 4 °C for 1 h, and the reaction was terminated by separating cells from buffer by centrifugation through a silicone/paraffin oil mixture as described (27). For inhibition and dis-placement experiments, cells were incubated with 125I-IL-8 prior to or after incubation with various concentrations of anti-Fy antibodies. Specific chemokine binding was determined by subtracting the cpm values obtained with Fy(a–b–) red cells and the mock cell line transfected or when an excess of unlabeled ligand was added to the binding reaction. An anti-RhD mAb was used as negative control of chemokine/antibody binding inhibition.

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anti-Fy at their saturating concentrations, but anti-Fy6 completely prevented IL-8 binding when used at 0.2× its saturating concentration. Similar IL-8 binding inhibition of the anti-Fy6 and anti-Fy3 mAbs was also observed when these experiments were performed with Chinese hamster ovary cells stably expressing the Fyα or Fyβ allelic form of DARC and when an anti-Fyβ human antiserum and Fy(a−b+) red cells were used (not shown). These results confirmed that the Fyα/Fyβ polymorphism, associated with a G42D substitution (9, 13, 26, 29) has no effect on the chemokine receptor properties of DARC (20, 29).

In the converse experiment, red cells were preincubated with various amounts of unlabeled IL-8 before anti-Fy mAbs were added. After a further incubation with a fluorescein isothiocyanate-conjugated second antibody, red cells were analyzed by flow cytometry. As shown in Fig. 2B, IL-8 inhibited in a dose-dependent fashion the binding of all anti-Fy mAbs but had no effect on the binding of the irrelevant anti-RhD mAb. A maximum of 60% inhibition of anti-Fy6 binding was reached with 500 nM IL-8, whereas 90–95% inhibition of anti-Fy3 and anti-Fyα binding was seen with IL-8 1.5 μM. Similar inhibition curves were obtained when the binding of anti-Fy mAbs was inhibited by preincubation of IL-8 at a constant concentration of 200 mM (data not shown).

Our results indicated that not only saturation of red cells with anti-Fyα, anti-Fy6, and anti-Fy3 antibodies, which represent large steric molecules, prevents IL-8 binding (4, 7, 8, 19, 20, 21), but also that preincubation of red cells with IL-8, a small polypeptide, interferes with anti-Fy mAb binding. This latter observation is in contradiction with the results of Hausman et al. (20). One possible explanation for this discrepancy might be that the IL-8 concentration used by this group (15–30 μM) was not high enough to completely saturate the chemokine receptor.
Fy6 Epitope Mapping by Biopanning of Phage Display Peptide Libraries and Site-directed Mutagenesis—To obtain information on amino acid residues involved in anti-Fy6 mAb binding, 6-residue and 15-residue phage display libraries were screened with the biotylated anti-Fy6 mAb (clone i3A). Insert sequences were determined from 60 phage clones selected by three rounds of biopanning on anti-Fy6 mAb (see “Results and Discussion”). Phagotopes isolated, the dipeptide FE, corresponding to residues 22 and 23 of the major isoform of DARC, was the most frequently represented (11 times, Table I).

To determine whether the dipeptide FE was part of the Fy6 epitope, F22V,E23A substitutions were introduced by site-directed mutagenesis in a recombinant vector carrying the cDNA encoding the major DARC isoform. The major and minor DARC isoforms encoded by the spliced and unspliced RNAs, respectively (26), differ only by the sequence of the six and eight NH2-terminal amino acids, respectively, and when expressed in K562 transfectants they bound the anti-Fy6 mAb (26) and IL-8, RANTES, and MGSA3 equally well. FACScan analysis of transfectants were stained with the anti-Fy3 mAb, which recognizes an epitope located in the third extracellular loop of DARC carrying the Fy6 and Fy3 epitopes most likely lie in very close proximity to each other. Examination of Fy6 reactivity indicated that MGSA binding to DARC transfectants was unaltered by anti-Fy3. This discrepancy remains unexplained. Altogether, displacement and inhibition experiments indicate that anti-Fya, anti-Fy6, and anti-Fy3 mAbs are all antagonists for IL-8 binding to red cells.

### Table I

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Irrelevant sequences 13


Glu23 motif should be involved in Fy6 expression. These results are in agreement with those reported by Wasniowska et al. (32) during the preparation of this manuscript and which demonstrated by the use of synthetic peptides/pins technology and deletion mutant analysis that the DFE residues, as central positions of the QLDFEDV heptapeptide comprising amino acid residues 19–25, are essential for the Fy6 epitope. Furthermore, Wasniowska et al. (32) also showed that all residues of the Fy6 epitope, except Phe 22 and Asp 24, could be replaced by one or two other amino acids without a significant decrease of antibody binding and that replacement of Glu 23 by 10 other amino acid residues gave a 2–3-fold increase of the mAb binding on synthetic peptides. This observation should account for the high frequency of the FS motif in the phage inserts that we have isolated with the anti-Fy6 mAb (Table I).

Chemokine Binding to the Phe22-Glu23 DARC Mutant—In parallel to Fy6 and Fy3 expression analysis, the wild type and Phe22-Glu23 mutant DARC transfectants were analyzed for binding of 125I-labeled chemokines. Nonspecific binding values were obtained with mock transfectants or when 200 nM unlabelled chemokines was added to the reaction mixture. Whereas radiolabeled IL-8, MGSA, and RANTES all bound to wild type transfectant cells, values corresponding to nonspecific background were obtained with the Phe22-Glu23 mutant (Fig. 3B).

These results indicated that alteration of the Fy6 epitope abolished the binding of CC and CXC chemokines to DARC. Together with the anti-Fy6/IL-8 competition experiments, these results provided the definitive proof of the tight relationship between the Fy6 epitope and the chemokine binding site on the NH2-terminal region of DARC.

Effect of Free Thiol Groups and Disulfide Bonds on Anti-Fy mAbs and Chemokine Binding—Inhibition of IL-8 binding to neutrophils pretreated by NEM, an alkylating reagent that primarily blocks sulfhydryl groups, suggested that free reactive sulfhydryl groups are present in the binding domain of the specific IL-8 type A receptor (22). Here, we investigated the effect of the treatment of red cells by NEM on the binding of the anti-Fy mAbs and IL-8 to DARC. Although 0.8 mM NEM inhibited IL-8 binding to neutrophils by 60% (22), incubation of red cells with increasing amount of NEM (0.2, 0.5, and 1 mM) for 30 min at 37 °C inhibited neither the binding of the anti-Fy mAbs (Fig. 4A) nor the binding of IL-8 (Fig. 4B). These results suggested that there is no free reactive sulfhydryl group on DARC participating in the binding site of chemokines or anti-Duffy mAbs.

It is known that on sodium dodecyl sulfate-polyacrylamide gel electrophoresis the DARC protein migrated as a 37- or 42-kDa species under reducing and nonreducing conditions,
respective, suggesting that cysteine residues of DARC may be involved in intrachain disulfide bond formation (29, 33). By analogy with the IL-8 receptors (23, 34, 35), it is assumed that the extracellular cysteines 51 and 276 and cysteines 129 and 197 (numbered on the major DARC polypeptide, see above) might be involved in disulfide bonds between the NH2-terminal domain and the third extracellular loop and between the first and second extracellular loops, respectively.

To determine whether the presence of disulfide bond(s) is critical for the antigenic and chemokine receptor properties of DARC, we analyzed the effect of DTT (100 mM) treatment of red cells and of cysteine substitutions on the binding of anti-Fy mAbs and chemokines (IL-8, RANTES, and MGSA). The efficiency of DTT treatment was monitored with an anti-Kell mAb (F18-7D6)4 which detects an epitope accessible only when intrachain disulfide bonds within the Kell glycoprotein are disrupted (30). Although DTT-treated cells reacted slightly less strongly with the three anti-Fy mAbs (predominantly with anti-Fy3), it is clear that intact disulfide bonds were not required for Fy4, Fy3, and Fy6 antigen expression (Fig. 4A). However, DTT treatment of red cells has a drastic negative effect on 125I-chemokine binding, especially for IL-8, the binding of which to DTT-treated red cells was reduced by 60% compared with untreated red cells (Fig. 4B).

Analysis of DARC mutants in which cysteines 51 and 54 or cysteine 276 was replaced by serine residues was in full agreement with the above results. Indeed, K562 cells stably transfected by the Cys356Ser mutants stained positively by the three anti-Fy mAbs, albeit less than cells expressing the wild type DARC (Fig. 5). Location of the Fy6 epitope within the third extracellular loop was deduced from antigen expression analysis of chimeric IL-8RB/DARC receptors (19). Fy6, Fy4, and Fy3 epitopes belong to a pocket defining the chemokine binding site and are brought into close proximity by a disulfide bridge between the first and fourth extracellular domains of DARC, as deduced from anti-Fy mAbs/chemokine binding competition analyses (4, 8, 19–21, and this report) and mutagenesis analysis (this report). The disulfide bond between the first and second extracellular loop is hypothetical.
amino acid residues that define the Fy6, Fyαb, and Fy3 epitopes (Fig. 6). It is expected that, together with the recent characterization of the Fy6 epitope (32, and our present results), the mapping of the Fyα and Fy3 epitopes will provide important clues for the characterization of the chemokine binding site on DARC.

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