The Duffy Antigen/Receptor for Chemokines (DARC) Is Expressed in Endothelial Cells of Duffy Negative Individuals Who Lack the Erythrocyte Receptor

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Summary

The Duffy antigen/receptor for chemokines (DARC), first identified on erythrocytes, functions not only as a promiscuous chemokine receptor but also as a receptor for the malarial parasite, *Plasmodium vivax*. The recent finding that DARC is ubiquitously expressed by endothelial cells lining postcapillary venules provides a possible insight into the function of this receptor because this anatomic site is an active interface for leukocyte trafficking. However, the biological significance of DARC is questionable since it has not yet been determined whether individuals lacking the expression of this protein on their erythrocytes (Duffy negative individuals), who are apparently immunologically normal, express the receptor on endothelial cells. However, we report here that DARC is indeed expressed in endothelial cells lining postcapillary venules and splenic sinusoids in individuals who lack the erythrocyte receptor. These findings are based on immunohistochemical, biochemical, and molecular biological analysis of tissues from Duffy negative individuals. We also present data showing that, in contrast to erythrocyte DARC, cells transfected with DARC internalize radiolabeled ligand. We conclude that the DARC may play a critical role in mediating the effects of proinflammatory chemokines on the interactions between leukocyte and endothelial cells since the molecular pathology of the Duffy negative genotype maintains expression on the latter cell type.

Chemokines are a family of proinflammatory molecules involved in immunoregulatory and inflammatory processes (1, 2) that specifically chemoattract and activate leukocytes (1). Chemokines can be divided into two families based on whether the first two cysteines are juxtaposed (C-C) or separated by a single amino acid residue (C-X-C) (2). In general members of the C-X-C branch, which include IL-8 and melanoma growth-stimulating activity (MGSA),[1] preferentially activate neutrophils, whereas C-C chemokines, which include RANTES and monocyte chemotactic protein 1 (MCP-1), exert more prominent effects on monocytes (2, 3).

The effects of chemokines on target cells are mediated through specific, high affinity receptors on the cell surface (4, 5). Although these specific receptors may bind more than one chemokine within the C-X-C or C-C family, they do not crossreact, i.e., the C-X-C receptors do not bind C-C chemokines and vice versa. However, we have recently characterized a promiscuous human erythrocyte chemokine receptor that binds both C-X-C and C-C chemokines with high affinity (6, 7). This receptor has subsequently been shown to be identical to the Duffy blood group antigen (8–10), which is the erythrocyte receptor for invasion by *Plasmodium vivax* (11). Individuals in West Africa, which is endemic for *P. vivax* infection, are negative for this blood group antigen, as are ~70% of African Americans (12).

Since we have recently shown that the Duffy antigen/receptor for chemokines (DARC) is expressed on endothelial

[1] *Abbreviations used in this paper:* DARC, Duffy antigen/receptor for chemokines; MCP-1, monocyte chemotactic protein 1; MGSA, melanoma growth-stimulating activity.

cells lining postcapillary venules (13), we reasoned that this receptor may be involved in leukocyte trafficking. However, the physiological relevance of DARC to leukocyte trafficking is unclear since individuals lacking expression of the Duffy red blood cell antigen seem to have normal immune functions. One possible explanation for these observations is that the major biological role of DARC is unrelated to its expression on erythrocytes and that it is the endothelial DARC that is more physiologically important. To address this issue we carried out experiments to determine whether DARC was expressed in the endothelial cells of Duffy negative individuals. Here we present data from immunohistochemical studies that confirm that DARC is expressed on the corresponding subset of endothelial cells of Duffy negative individuals. This is further supported by biochemical analysis of membranes from the spleen of a Duffy negative individual which confirmed the expression of DARC. Investigations of receptor function using transfectants revealed that DARC bound and internalized ligand, whereas the latter function could not be demonstrated in erythrocytes. Overall these findings suggest that DARC is involved in mediating the effects of proinflammatory chemokines on endothelial cells lining postcapillary venules.

Materials and Methods

Materials. ^{125}I-IL-8 and ^{125}I-MGSA (specific activity 2,200 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Unlabeled IL-8 and MGSA were purified as previously described (6, 14). RANTES and MCP-1 were from Peprotech (Princeton, NJ). Enriched human erythrocytes from outdated blood were obtained from Peninsula Blood Bank (Burlingame, CA). Reagents for electrophoresis were from Bio-Rad Labs. (Richmond, CA) and FMC Corp. (Rockland, ME). All other reagent grade chemicals were from Sigma Chemical Co. (St. Louis, MO). The Fy6 mAb to the Duffy blood group antigen was kindly provided by Dr. M. Nichols (Abbott Labs., Chicago, IL) as a hybridoma culture supernatant containing 22 μg per ml of IgG (15).

Immunohistochemistry. Tissue blocks were obtained from the files of the University of Louisville Hospital. Sections were cut at 6 μm, deparaffinized with xylene, and rehydrated with ethanol. After washing with PBS, slides were incubated with Fy6. Binding of the monoclonal antibody was detected by sequential incubations with a biotinylated secondary antibody, followed by complexes of avidin-biotinylated peroxidase. The reaction product was localized by incubation with the diaminobenzidine substrate.

Procurement of Human Tissue. Human tissues were obtained at the University of Louisville Hospital according to a standard protocol by which the operative consent form includes permission that the tissue can be used for research purposes after being thoroughly examined by the pathologist responsible for diagnostic evaluation of the specimen.

Isolation of Erythrocytes and Production of Erythrocyte Ghosts. Human erythrocytes were isolated from whole blood using standard techniques (16). Erythrocyte ghosts were prepared as described previously (6).

Preparation of Cell Membranes. Human spleen from a Duffy negative individual (3.5 g) was homogenized in 50 mM Tris-HCl buffer, pH 7.4, containing 5 μg/ml each of leupeptin and aprotinin, 0.1 mM PMSF, 0.05 mM Pefabloc, and 1 mM EDTA (lysis buffer). The homogenate was centrifuged at 500 *g* for 20 min. The pellet, which consisted of cell debris and nuclei, was discarded and the supernatant was centrifuged at 48,000 *g* for 30 min. The resulting pellet, which consisted of total cell membranes was removed and resuspended to a final concentration of 5.5 mg per ml in lysis buffer and stored at −20°C until further use.

^{125}I-Labeled Chemokine Binding to Duffy Negative Spleen Membranes. Human spleen membranes from a Duffy negative individual (50 μg) were incubated with ^{125}I-labeled MGSA (0.5 nM) and varying concentrations of unlabeled MGSA at 4°C for 1 h in PBS, pH 7.4. Binding was stopped by filtration through Whatman GF/C filters pretreated with 1% polyethyleneimine (Whatman Inc., Clifton, NJ). Filters were rinsed three times with 2 ml of ice-cold PBS and counted in a gamma counter (Iso-Data 100; Isodata, Costa Mesa, CA). Nonspecific binding was determined in the presence of 100 nM unlabeled ligand. Binding data were analyzed by the Ligand program (17) as modified for the IBM PC (18).

Internalization of ^{125}I-labeled MGSA in K562 Cells Transfected with the DARC cDNA. 10^6 cells per ml were incubated with ^{125}I-labeled MGSA in the presence and absence of unlabeled MGSA at 4°C and at 37°C for the designated times. Aliquots were removed from each tube and the suspension centrifuged at 12,000 *g* for 1 min to sediment the cells. After discarding the supernatants the cell pellets were resuspended either in ice-cold PBS or in acidic media (PBS, pH 3) for 2 min. The incubation was terminated by removing aliquots from the cell suspension and separating cells from buffer by centrifugation through a silicon/paraffin oil mixture as described previously (19). Non-specific binding was determined in the presence of 1 μM unlabeled MGSA. Treatment of cells incubated at 4°C with acidic buffer resulted in the displacement of >95% of the bound counts and yielded an estimate of total cell surface binding. Cell-associated radioactivity was then determined by acid extraction of cells at 37°C which removed the cell surface-bound material and thus gives a measure of the internalization of the ligand.

Immunoblot Analysis. Proteins were subjected to electrophoresis in 12% Novex precast minigels and transferred electrophoretically to Problot (Applied Biosystems Inc., Foster City, CA) in 10 mM CAPS (3-[cyclohexolamino]-1-propane sulfonic acid), pH 11, containing 10% methanol for 1 h at a current of 250 mA. After transfer, the blots were incubated for 60 min at room temperature in 25 mM Tris–HCl, pH 7.4, containing 150 mM NaCl and 0.1% Tween 20. The blots were then incubated overnight at 4°C with a 1:1,000 dilution of Fy6. The blots were washed several times in Tris buffer and subsequently incubated in a 1:20,000 dilution of horseradish peroxidase-conjugated goat anti-mouse antibody (Tago Inc., Burlingame, CA) for 1 h at room temperature. After extensive washing, the blots were developed by ECL, according to the manufacturer's instructions (Amersham Corp., Arlington Heights, IL).

Cross-linking of ^{125}I-MGSA to Cell Membranes. Aliquots of the particulate membrane function (150 μg) were incubated in the presence of 5 nM of ^{125}I-MGSA, in the presence or absence of 1 μM unlabeled MGSA, for 1 h at 4°C in PBS, pH 7.4. At the end of the incubation, the membranes were pelleted by centrifugation (100,000 *g*, for 15 min), made up to the original volume in PBS, and chemically cross-linked with EDC (1, ethyl-3-[3-dimethaminopropyl]carbodiimide hydrochloride), at a final concentration of 1 mM for 1 h at room temperature. The membranes were then pelleted as described above and solubilized in SDS sample buffer in the presence of 50 mM dithiothreitol (DTT) for 3 min at room temperature and then analyzed by electrophoresis in 12% Tris-glycine polyacrylamide gels containing SDS.

Northern Blot Analysis. Pieces of human spleen were extracted with guanidinium isothiocyanate (20) and total cellular RNA was

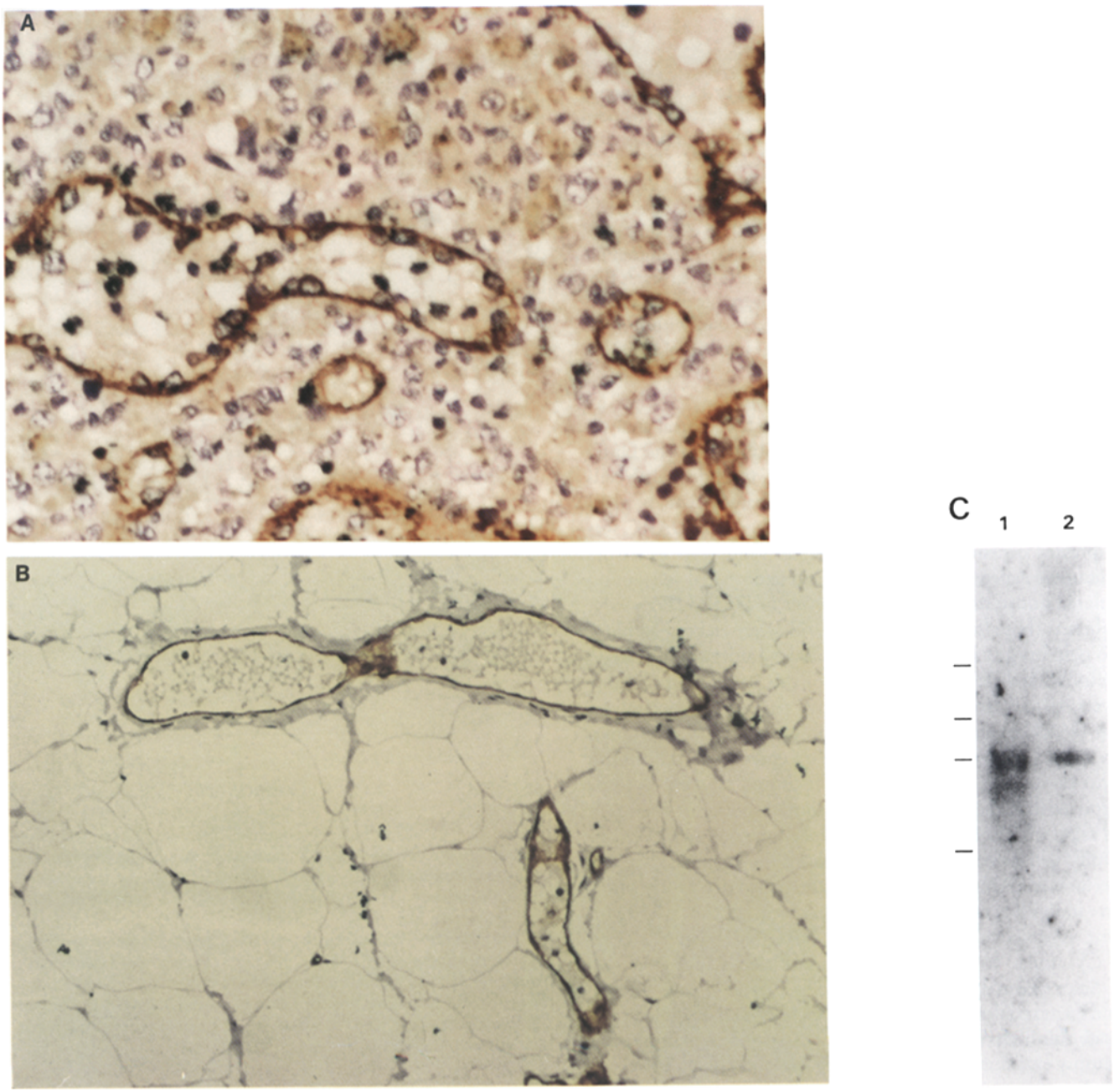

Figure 1. Immunohistochemical localization and Northern blotting of DARC in endothelial cells of postcapillary venules in spleen. (*A*) DARC is expressed by endothelial cells lining sinusoids of splenic red pulp. The spleen from a Duffy positive individual was stained with Fy6 and counterstained with hematoxylin (×400). There is strong immunoreactivity with specialized endothelial cells lining the sinusoids. Hemosiderin-laden macrophages in the background have an amber color. (*B*) Endothelial cells lining postcapillary venules in Duffy negative individuals express DARC. Subcutaneous tissues from a Duffy negative woman who underwent mastectomy for mammary carcinoma were analyzed for DARC expression by immunohistochemistry. Paraffin-embedded tissues were processed as described under experimental procedures. Endothelial cells lining thin-walled venules demonstrated high level expression whereas erythrocytes in the lumena of the vessels lacked immunoreactivity. (*C*) Spleen from Duffy negative individuals expresses DARC mRNA transcripts. Total RNA was extracted from the spleen of a Duffy negative individual who underwent autopsy examination (lane *1*) and the human erythroleukemia (*HEL*) cell line (lane *2*) and separated by size in agarose gels containing formaldehyde. Northern blotting analysis with a radiolabeled DARC cDNA probe demonstrated that both RNA samples contained a mRNA transcript of ~1,300 bp. The mobility of RNA of standard size is indicated on the left (from top, 4,400, 3,370, 1,350, and 240 bp).

quantitated by measuring the absorbance at 260 nm. Aliquots of RNA were subjected to electrophoresis in a 1.2% agarose gel containing 20 mM MOPS, 5 mM sodium acetate, 5 mM EDTA, and 6% formaldehyde. The size-fractionated RNA was transferred to a nylon membrane. A cDNA probe spanning the coding sequences of DARC (21) was labeled with α-[^{32}P]dCTP (3000 μCi/mMol) using random hexanucleotide primers. The probe was allowed to hybridize to the membrane overnight at 42°C in the presence of

50% formamide. Blots were washed under stringency conditions (55°C in 0.1× SSC, 0.1% SDS).

Nucleotide Sequence Analysis of the DARC Gene. Segments of the gene encoding DARC (21) were amplified by PCR. Genomic DNA served as templates in standard PCR amplification reactions containing sense and antisense oligonucleotide primers designed from untranslated sequences upstream (5'TTCCCAGGAGACTCTTCCGGT) and downstream (5'ACTTTAATTCAGGTTGACAGGTGGG) of the exon encoding the DARC polypeptide, respectively. The resulting amplification product of ~1,100 bp was subcloned into the TA vector (Invitrogen, San Diego, CA). The nucleotide sequence was determined using a Sequenase kit (United States Biochemicals Corp., Cleveland, OH) with universal forward and reverse primers that anneal to sites in the vector and with primers corresponding to DARC-coding sequences at intervals of ~300 bp.

Results and Discussion

We previously showed, by immunohistochemical analysis of archival tissues and a monoclonal antibody to the Duffy antigen (15), the expression of DARC to endothelial cells lining small, thin-walled blood vessels characteristic of post-capillary venules in the kidney (13). Furthermore DARC was expressed on endothelial cells of small venules in all tissues examined except for liver. Specialized endothelial cells lining splenic sinusoids expressed high levels of DARC (Fig. 1 *A*), whereas those lining hepatic sinusoids were lacking activity. To gain insight into the physiological significance of DARC in individuals lacking expression of this protein on erythrocytes, tissues from a Duffy negative patient were examined for DARC expression by immunohistochemistry. As shown in Fig. 1 *B*, DARC was expressed on endothelial cells lining post-capillary venules present in soft tissues, whereas erythrocytes within the lumen failed to show binding of the mAb as expected. To further examine the expression of DARC in Duffy negative individuals, Northern blotting analysis was performed. RNA extracted from the spleen of a Duffy negative individual contained an mRNA transcript of ~1,300 bp that annealed to a radiolabeled DARC cDNA probe (Fig. 1 *C*) similar to that expressed in HEL cells (22).

Since spleen expressed high levels of DARC, ligand binding and immunoblotting experiments were carried out to corroborate this observation using splenic tissue obtained from the autopsy examination of a Duffy negative individual. Receptor binding asssays using membrane fractions prepared from the Duffy negative spleen demonstrated a promiscuous chemokine binding profile characteristic of DARC (4); ^{125}I-MGSA binding was completely displaced by excess unlabeled MGSA, IL-8, RANTES, and MCP-1. As expected, this spectrum of chemokine binding, which is not a feature of the other four known chemokine receptors (i.e., type A or B IL-8 receptor, RANTES/MIP-1α receptor, or the MCP-1 receptor) (23–26), was inhibited by the Fy6 monoclonal antibody to DARC, but not by a monoclonal antibody to the type A IL-8 receptor (Fig. 2 *A*). Scatchard analysis of ^{125}I-MGSA binding (Fig. 2 *B*) revealed high affinity binding, similar to that of DARC expressed by (Duffy positive) erythrocytes (6, 7) and (renal) endothelial cells (13), K_D ~3 nM.

Additional characterization of this receptor was undertaken by chemical cross-linking experiments using the Duffy negative

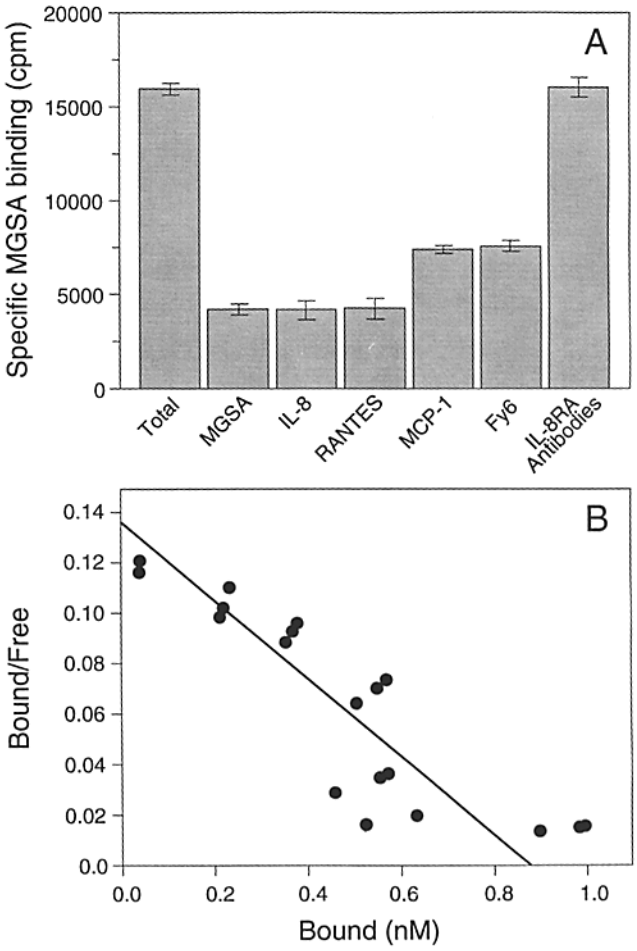

Figure 2. ^{125}I-MGSA binding to human spleen membranes. (*A*) Inhibition of ^{125}I-MGSA binding to human spleen membranes. Membranes (50 μg) were incubated with 500 pM radiolabeled MGSA in the presence of 100 nM unlabeled MGSA, IL-8, RANTES, MCP-1, and MIP-1α. The binding reactions were stopped as described under Materials and Methods. Data are from three separate experiments and the error bars show the SEM. (*B*) Scatchard analysis of ^{125}I-MGSA binding to human spleen membranes. Membranes (50 μg) were incubated with 500 pM radiolabeled MGSA in the presence of increasing concentrations of unlabeled MGSA at 4°C for 1 h. Binding was terminated by filtration and counted as described under Materials and Methods.

splenic membrane fractions previously used for the ligand binding assays (Fig. 3). Cross-linking with radiolabeled MGSA revealed a labeled protein with a molecular mass of 48 kD. Assuming a molecular mass of 8 kD for MGSA and one ligand molecule per receptor, a molecular mass of 40 kD is deduced for the receptor. This is consistent with the reported molecular mass of DARC (6, 7, 13). Since ligand binding activity of the receptor was blocked by Fy6, immunoblotting was performed to confirm the molecular mass of this protein (Fig. 4). A protein of similar molecular mass (~40 kD) is evident in lanes containing membrane fractions from spleen and erythrocytes (RBC).

To gain insight into the mechanism responsible for DARC expression in Duffy negative individuals, the genomic locus encoding DARC was amplified from DNA templates using primers designed from sequences in the 5′ and 3′ untrans-

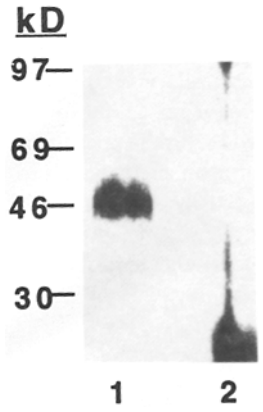

Figure 3. Covalent cross-linking of ^{125}I-MGSA to human spleen membranes. Membranes prepared from human spleen (150 μg) were incubated with 5 nM ^{125}I-MGSA in the absence (lane *1*) and presence (lane *2*) of 1 μM unlabeled MGSA. After 1 h at 4°C, the membranes were washed free of unbound label. The bound ^{125}I-MGSA was cross-linked by incubation with EDC for 1 h at room temperature and then washed with PBS before analysis by SDS-PAGE. Aliquots of 150 μg of protein were applied to the gel. After electrophoresis the gels were dried down and subjected to autoradiography.

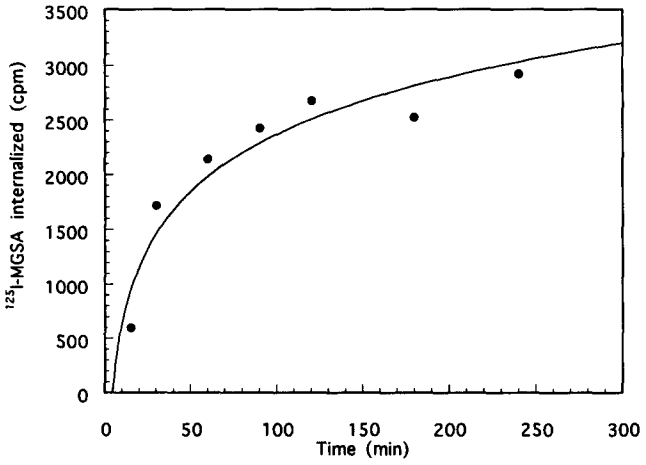

Figure 5. The time course of internalization of ^{125}I-MGSA by K562 cells transfected with DARC. Cells were incubated with ^{125}I-MGSA at 37 and 4°C. Aliquots were removed at the times indicated, cooled to 4°C, and acid stripped with PBS, pH 3, for 2 min to dissociate surface bound ligand. Bound ligand was separated from free by centrifugation through oil as described above. Data are expressed as cpm of ^{125}I-MGSA that were internalized. Internalized ligand was calculated by subtracting specific binding in cells (incubated at 37°C) that were acid stripped from those that were not. Acid stripping of cells incubated at 4°C decreased specific binding by greater than 95% which is a direct measure of the efficiency of this method. Data shown are representative from two separate studies.

lated regions of RNA transcripts by PCR. Electrophoretic analysis of PCR products revealed an identical size to that obtained when mRNA was reverse transcribed and cDNA served as templates in PCR reactions with the same primers. Nucleotide sequence analysis of amplification products from the genomic templates from a Duffy positive individual revealed that the polypeptide was encoded by a single, uninterrupted exon. Parallel nucleotide sequence analysis of multiple clones of DNA amplification products from a Duffy negative individual did not reveal any differences in coding sequence compared to that of a Duffy positive individual. Nucleotide sequence analysis of multiple clones revealed two sequences that differed only in the second nucleotide of codon 44. Approximately half of the clones from the Duffy positive individual analyzed contained GAT and the remainder contained GGT, which encode aspartic acid and glycine residues, respectively. These differences in sequence could constitute the difference between the Duffy A and B blood groups.

In common with other cloned chemokine receptors (5), genomic sequences encoding DARC lack introns. Since the predicted primary structure of DARC is identical in Duffy positive and negative individuals it is concluded that the mechanisms responsible for silencing the expression of DARC in erythroid cells are not related to a defect in the coding sequence. Since mRNA transcripts are absent from erythroid elements in Duffy negative individuals (21), we speculate that this mechanism may involve *cis*-acting sequences that control transcription of this gene in erythroid cells. This has been previously described in selected cases of hereditary elliptocytosis in which isoform-specific mutations result in complete deficiency of protein 4.1 in erythrocytes but not nonerythroid cells (27).

Taken together these immunohistochemical, biochemical, and genetic data establish that DARC is expressed by endothelial cells lining postcapillary venules in Duffy negative individuals. The receptor demonstrated a promiscuous chemokine binding profile and had the same molecular weight described for DARC. These findings suggest that a DARC protein identical to that present in Duffy positive individuals is expressed on endothelial cells when the negative selection from morbidity and mortality from *P. vivax* resulted in the abrogation of expression of this protein by erythrocytes. This dichotomous expression in Duffy negative individuals raises the possibility that DARC plays an essential role in the biology of endothelial cells, whereas its function on erythrocytes appears to be dispensable.

At least four formal biologic mechanisms of action suggest themselves for this receptor. They include: (*a*) a signaling receptor, (*b*) a chemokine transporter, (*c*) an accessory, presenting receptor, and (*d*) a sink for the inactivation of chemokines. Preliminary experiments were carried out to explore these possibilities. One feature that characterizes both signaling receptors and chemokine transporters is the internalization of bound ligands. The ability of DARC K562 transfectants to internalize radiolabeled chemokines is shown in Fig. 5. Rapid internalization of ^{125}I-MGSA is evident and by

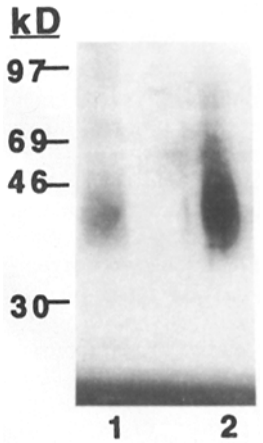

Figure 4. Western blot analysis of human spleen membranes. Cell membranes (150 μg) from a Duffy negative spleen (lane *1*) and Duffy positive erythrocyte ghosts (lane *2*) were subjected to SDS-PAGE, transferred electrophoretically to Problot, and analyzed as indicated under Materials and Methods.

3 h, almost 40% of the bound label is internalized. SDS-PAGE analysis of the internalized chemokine over time revealed that it remained intact (data not shown). Collectively, these functional studies suggest that DARC may have a signaling and/or transporter-like role, but that it lacks features associated with a ligand-presenting receptor subunit. Whereas Duffy positive erythrocytes were shown to function as a sink for ligand this may not reflect the central physiological significance of the receptor because the absence of this function in Duffy negative individuals is not associated with any apparent abnormality of chemokine activity. The findings presented here demonstrate the preservation of DARC expression by endothelial cells lining postcapillary venules and splenic sinusoids in individuals negative for the Duffy blood group antigen, who could be considered Nature's experiment that abrogated expression of this receptor on erythrocytes to provide protection from malaria induced by *P. vivax*. This suggests that DARC is a biologically relevant receptor that could play a key role in inflammation and leukocyte trafficking at these active interfaces of circulation.

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