Activation State of α4β1 Integrin on Sickle Red Blood Cells Is Linked to the Duffy Antigen Receptor for Chemokines (DARC) Expression*

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In sickle cell anemia, reticulocytes express enhanced levels of α4β1 integrin that interact mainly with vascular cell adhesion molecule-1 and fibronectin, promoting vaso-occlusion. These interactions are known to be highly sensitive to the inflammatory chemokine IL-8. The Duffy antigen receptor for chemokines (DARC) modulates the function of inflammatory processes. However, the link between α4β1 activation by chemokines and DARC erythroid expression is not or poorly explored. Therefore, the capacity of α4β1 to mediate Duffy-negative and Duffy-positive sickle reticulocyte (SRe) adhesion to immobilized vascular cell adhesion molecule-1 and fibronectin was evaluated. Using static adhesion assays, we found that, under basal conditions, Duffy-positive SRe adhesion was 2-fold higher than that of Duffy-negative SRes. Incubating the cells with IL-8 or RANTES (regulated on activation normal T cell expressed and secreted) increased Duffy-positive SRe adhesion only, whereas Mn\textsuperscript{2+} increased cell adhesion independently of the Duffy phenotype. Flow cytometry analyses performed with anti-β1 and anti-α4 antibodies, including a conformation-sensitive one, in the presence or absence of IL-8, revealed that Duffy-positive and Duffy-negative SRes displayed similar erythroid α4β1 expression levels, but with distinct activation states. IL-8 did not affect α4β1 affinity in Duffy-positive SRes but induced its clustering as corroborated by immunofluorescence microscopy. Our results indicate that in Duffy-negative SRes α4β1 integrin is constitutively expressed in a low affinity state, whereas in Duffy-positive SRes α4β1 is expressed in a higher chemokine-sensitive affinity state. This activation state associated with DARC RBC expression may influence the intensity of the inflammatory responses encountered in sickle cell anemia and participate in its interindividual clinical expression variability.

α4β1 (CD49d/CD29, VLA4), the only integrin expressed on young SRes,\textsuperscript{3} is a multifunctional adhesion protein mediating cell attachment via different ligands, the majors being VCAM-1 and FN (1, 2). In addition to reduced deformability of sickle erythrocytes, increased red cell adhesion to endothelium has been reported (3, 4). This abnormal adhesion may profoundly influence vaso-occlusive crises, by favoring cell contacts with the microvascular and venular endothelium via the α4β1/VCAM-1 and/or the α4β1/FN interactions (2, 5–7). As demonstrated previously, these interactions are highly sensitive to the inflammatory chemokine IL-8 in SCA (6, 7) as in other situations (8). In addition, the implication of IL-8 in the severity of vaso-occlusive episodes has been demonstrated (9). Together, these data suggest the importance of IL-8 in vaso-occlusive crises through α4β1 activation (5–7, 9, 10).

Red blood cells (RBCs) express the Duffy blood group antigens (Fy) (11) carried by a glycoprotein identified as the Duffy antigen receptor for chemokines (DARC) of both the CC and the CXC classes, including IL-8 and RANTES (12). Four major Duffy phenotypes are serologically identified: Fy (a+b−), Fy (a+b+), Fy (a−b+) (Duffy-positive) and Fy (a−b−) (Duffy-negative); although the latter is predominant in the populations of African descent, it is rare in Caucasians (11). Originally described as a binding site for Plasmodium vivax and Plasmodium knowlesi (12), DARC is known to serve as a sink for the clearance of chemokines from the circulation (13). However, DARC is also described as a blood reservoir by sustaining chemokine blood levels (14). Therefore, DARC may mitigate the inflammatory response by modulating the cellular activation mediated by chemokines. The IL-8-mediated activation of the α4β1/VCAM-1 and the α4β1/FN interactions in SCA (6, 7) has not been evaluated in association with DARC expression on RBCs. Recently, Nebor et al. (15) demonstrated that Duffy-positive SCA patients exhibited higher plasma levels of IL-8 and RANTES than Duffy-negative patients.

\textsuperscript{3}The abbreviations used are: SRe, sickle reticulocyte; DARC, Duffy antigen receptor for chemokines; FN, fibronectin; MFI, mean fluorescence intensity; RANTES, regulated on activation normal T cell expressed and secreted; SCA, sickle cell anemia; VCAM-1, vascular cell adhesion molecule-1.
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Considering that these interactions could be important targets for therapeutic strategies in this disease (2), it is important to evaluate the effect of the Duffy phenotype on α4β1-mediated SRe adhesion to immobilized VCAM-1 and FN. To this end, static adhesion assays, which may reflect the low in vivo flow conditions (16), were performed in the presence or absence of IL-8 or RANTES, also known to activate the α4β1/VCAM-1 interaction (8, 17). Adhesion assays were also performed in the presence of Mn2+ and the activating anti-β1 antibody TS2/16, both known to induce a high affinity state of α4β1 (18). Modifications in α4β1 adhesive properties are regulated either by conformational changes (affinity changes) or by avidity changes (clustering) (8, 19). Both aspects were investigated by flow cytometry and immunofluorescence staining in Duffy-negative and Duffy-positive SRes, using anti-β1 and anti-α4 antibodies.

We demonstrated that, in Duffy-negative SRes, α4β1 was expressed in a low affinity state that may be switched toward a high affinity state by Mn2+ or anti-β1 TS2/16 but not by IL-8 or RANTES, whereas in Duffy-positive SRes, α4β1 is constitutively expressed in a higher IL-8/RANTES-sensitive affinity/avidity state. We hypothesize that this activation state associated with DARC RBC expression may influence the intensity of the inflammatory responses encountered in this pathology and participate in its interindividual clinical expression variability.

EXPERIMENTAL PROCEDURES

Patients—Two healthy controls (AA) and 33 SCA adult patients (SS patients) who regularly attended the local sickle cell center of Guadeloupe, a French West Indies island, were included in this study. Patients did not show obvious signs of infection, were not pregnant or undergoing hydroxyurea or transfusion. Informed written consent was secured from all patients before any sample of blood was obtained. This study was approved by the local ethical committee. Quantitative DARC RBC expression phenotype was determined from restriction fragment length polymorphism genotyping performed by a PCR-based approach as described previously by Yazdanbakhsh et al. (20) and Chaudhuri et al. (21). Our cohort of patients was composed of 18 Duffy-negative patients (Fy (a-b-)) and among the Duffy-positive patients, 4 presenting an expression level of DARC of 100% ((Fy (a+b+)) and 6 and 5, respectively, an expression level of 50% (Fy (a+b-) or Fy (a-b-)).

Blood Samples and Density Fractionation—Whole blood samples were obtained by venipuncture into EDTA Vacutainer tubes from healthy normal donors and SS patients during regular visits. The sickle RBC suspension was enriched in reticulocytes using a discontinuous-density gradient of Percoll (GE Healthcare) with densities (d) 1.076 and 1.106. The sickle RBC suspension enriched in reticulocytes (1.076 < d ≤ 1.106) was washed three times in buffer A (140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1 mM NaH2PO4-Na2HPO4 (20:80), 10 mM glucose, and 10 mM HEPES-Tris (pH 7.4 at 37 °C, 300 mosmol/kgH2O)) and stored at 4 °C. Automated SRe quantitation (%) was performed using the Sysmex XT-2000i automatic cell counter (Sysmex) or using thiazole orange dye (Retic-count; Becton-Dickinson) and a BD FACSCanto II flow cytometer (Becton-Dickinson) with FACSDiva software (version 6.1.2) for acquisition and analysis. All experiments were conducted with the sickle RBC suspension enriched in reticulocytes from Duffy-negative or Duffy-positive patients: Duffy-negative SRes and Duffy-positive SRes. Just before the experiments were performed, the cells were washed once in buffer B (140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 10 mM glucose, and 10 mM HEPES-Tris (pH 7.4 at 37 °C, 300 mosmol/kgH2O)).

Treatment with IL-8, RANTES, Anti-β1 TS2/16, Anti-α4 HP2.1, and Mn2+—Prior to adhesion assays, Duffy-negative and Duffy-positive SRes, resuspended at 15% hematocrit in buffer B, were preincubated for 30 min at 37 °C with 100 ng/ml IL-8 (Sigma) or 100 ng/ml RANTES (Sigma) and washed at the end of the incubation in buffer C (25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM KCl, 10 mM glucose) to be resuspended at 2% hematocrit (2 × 10⁶ cells/ml) in buffer D (buffer C supplemented with 1 mM MgCl2 and 1 mM CaCl2, 0.5% heat-denatured BSA, 5 μg/ml human transferrin (Sigma), and 5 μg/ml bovine insulin (Sigma)). For integrin activation through Mn2+, Duffy-negative and Duffy-positive SRes were washed once with buffer C and resuspended at 2% hematocrit, in buffer E (buffer C supplemented with 2 mM MnCl2, 0.5% heat-denatured BSA, 5 μg/ml human transferrin (Sigma) and 5 μg/ml bovine insulin (Sigma)). For activation assays through anti-β1 TS2/16 (Santa Cruz, Biotechnology), Duffy-negative and Duffy-positive SRes, directly diluted at 2% hematocrit in buffer D, were incubated with 2 μg/ml anti-β1 TS2/16 for 20 min on ice. For inhibition assays, cells were diluted at 2% hematocrit in buffer D or E and incubated for 30 min on ice with 20 μg/ml anti-α4 HP2.1 (Santa Cruz Biotechnology) or 20 μg/ml isotype-matched antibody used as control.

Static Adhesion Assays—Duffy-negative SRes, Duffy-positive SRes and control AA adherence, performed under static conditions, was evaluated by a colorimetric method based under hemoglobin absorbance at 540 nm after lysis of the RBCs specifically attached. Briefly, 96-well flat-bottomed microtiter plates (NUNC) were coated in quadruplicate with human sVCAM-1 (R&D Systems) at 0.5 μg/ml or human FN (Sigma) at 8 μg/ml by overnight incubation at 4 °C, followed by saturation of free plastic sites of wells with heat-denatured BSA (5%) for 2 h at 37 °C. Just before adhesion, plates were washed either with buffer D or buffer E. Then, 100 μl of control AA (2% hematocrit) or 100 μl of untreated or treated Duffy-negative SRes or Duffy-positive SRes (2% hematocrit) in the respective buffer was added to each well and allowed to sediment for 5 min at 4 °C and then placed at 37 °C for 90 min, a sufficient time to achieve maximum adhesion. Nonadherent cells were removed by inverting the plate on Whatman paper and washing them gently twice with the respective warm buffer (250 μl). The adherent cells were lysed with 150 μl/well Drabkin’s reagent (Sigma) prepared as described by the manufacturer. Background adhesion was assessed with heat-denatured BSA wells and was <5% of the total.

Cell adhesion was determined as follows. After lysis in Drabkin’s reagent, the amount of hemoglobin released in each
well was quantified at 540 nm, using a TRIAD series multimode detector (Dynex Technologies), the color intensity being proportional to the hemoglobin released and, in inference, to the number of cells attached in each well. In parallel, 100 μl of Duffy-negative and Duffy-positive SRes (2 × 10⁶ cells) were centrifuged, the pellets were lysed with 150 μl of Drabkin’s reagent (Sigma) and the absorbance read at 540 nm, these values representing 100% of RBCs added in each well, in inference, 100% of SRes added in each well. Therefore, all absorbance measurements were normalized for this latter quantified by the Sysmex XT-2000i automatic cell counter.

Adhesion percentage was calculated as follows: number of adherent SRes (A₅₄₀ (lysate))/total SRes added (A₅₄₀ (lysate)) × 100, and the means ± S.D. of quadruplicate samples were calculated.

**Flow Cytometry**—Expression levels of α4β1 integrin were measured by flow cytometry with monoclonal antibodies using indirect staining as described previously (22). Briefly, untreated or IL-8-treated SRes were prepared as described previously. Cells were then resuspended with isotype controls IgG (5 μg/ml) or HP2.1 (Santa Cruz Biotechnology), 9F10 (Pharningen BD Biosciences), 4B4 (Beckman Coulter), MAR4, CD45-APC (Pharmingen BD Biosciences) at 5 μg/ml and anti-Fy6 antibody (23) (NaM185-2C3 clone, Duffy-specific) diluted at 1/10 (crude supernatant) at 4 °C for 1 h in a PBS/0.2% (v/v) BSA (Sigma) solution pH 7.2. After primary incubation, cells were washed twice in wash buffer and incubated in the dark at 4 °C for 1 h with secondary R-phycocerythrin antibody (Beckman Coulter) at a concentration of 5 μg/ml in PBS/0.2% BSA solution and with 20 μl of CD45-APC to exclude residual white blood cells. Percentage of reticulocytes in fractionated blood samples was determined using thiazole orange dye (Retic-Count™; Becton Dickinson). The specific antibody-binding capacity per erythroid cell, which is an estimate of the copy number of antigen molecules/cell, was deduced from a calibration curve obtained with Qifikit calibration beads (Dako). Flow cytometric analyses were performed on BD FACs Canto II flow cytometer® (BD Biosciences) using the BD FACS Diva software (version 6.1.2) for acquisition and FlowJo version 7.2.5 (Treestar, Inc.) for analysis. Instrument setup and calibration were performed daily with CST beads according to the manufacturer’s recommendations. A forward scatter threshold was set to eliminate debris from list mode data and for each sample. The light scatter (side scatter and forward scatter) and the fluorescence channels were set on a logarithmic scale, a minimum of 10,000 reticulocytes being analyzed in each condition.

**Indirect Immunofluorescence Microscopy**—Clustering of α4β1 was analyzed by indirect immunofluorescence (24). Briefly, Duffy-positive and Duffy-negative SRes were pre-treated with IL-8, RANTES, or Mn²⁺ as described above. Immediately, after this treatment, aliquots of 5 × 10⁶ cells were washed in the respective ice-cold buffers, then incubated on ice for 30 min in the presence of anti-α4 HP2.1 (20 μg/ml). After washing with ice-cold buffer, cells were further incubated on ice in the presence of a 1:40 dilution of a rabbit fluorescein isothiocyanate (FITC) anti-mouse IgG (Dako). Non-specific binding was assessed by cells exposed to the latter antibody alone. Washed Duffy-positive SRes and Duffy-negative SRes were fixed with 1% paraformaldehyde and spotted on glass slides using a conventional cytosin centrifuge (Shandon Southern Instruments) to be mounted for indirect immunofluorescence observation. Cells were viewed under an inverted-phase fluorescence ECLIPSE 80i photomicroscope with a Nikon Digital camera DXM 1200F and an ACT-1 acquisition software (Nikon).

**Statistical Analysis**—Statistical analysis of the data related to the effects of the various treatment conditions was accomplished using a two-way analysis of variance (ANOVA) followed by Wilcoxon matched-paired test. The differences between Duffy-negative SRes and Duffy-positive SRes were compared by mean of nonparametric Mann-Whitney tests. A p < 0.05 was considered significant. The analysis was performed with the statistical software GraphPad Prism version 5.0 for Windows (GraphPad Software).

**RESULTS**

**Greater Adhesion of Duffy-positive SRes to Coated FN and VCAM-1**—Our important prerequisite in this study was to compare, under basal conditions, adhesion of Duffy-negative and Duffy-positive SRes on coated FN and VCAM-1, two specific α4β1 ligands. In static adhesion assays, SRE adhesion onto coated FN or coated VCAM-1 was similar among all Duffy-positive phenotype groups, Fy(a+b−), Fy(a+b+) and Fy(a−b+) (Fig. 1A). Thus, for the following comparative experiments with Duffy-negative SRes the data of Duffy-positive SRes were averaged. Surprisingly, Duffy-negative SRE adhesion was significantly lower onto FN (3.49 ± 0.66%; p = 0.0002) and VCAM-1 (3.43 ± 0.43%; p = 0.0004) than that of Duffy-positive SRe (6.47 ± 1.08% and 6.59 ± 0.84%, respectively) (Fig. 1B). Control AA RBCs did not adhere to the coated ligands (data not shown) as was shown previously (2–4). Unlike the isotype-matched control antibody, anti-α4 HP2.1-blocking antibody reduced both Duffy-negative and Duffy-
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FIGURE 2. Effects of chemokines, activating TS2/16, and Mn2+ on Duffy-negative and Duffy-positive SRe adhesion. Static adhesion assays to 8 μg/ml FN and 0.5 μg/ml VCAM-1 were assessed as described under “Experimental Procedures.” Adhesion percentage is calculated as above. A, neither 100 μg/ml IL-8 nor 100 μg/ml RANTES treatments affected adhesion of Duffy-negative SRes (n = 18). B, chemokine-treated Duffy-positive SRes (n = 15) were 2-fold more adherent than the untreated cells to FN (**, p < 0.001; ***, p < 0.01) and VCAM-1 (**, p < 0.01). Pretreatment with the blocking anti-α4 HP2.1 antibody (20 μg/ml) inhibited adhesion. C, activating anti-β1 TS2/16 antibody (2 μg/ml) and 2 mM Mn2+ induce a 6-fold increased adhesion of Duffy-negative SRes to FN (**, p < 0.05 and ***, p < 0.01, respectively) and to VCAM-1 (***, p < 0.01). Pretreatment with the blocking anti-α4 HP2.1 antibody (20 μg/ml) reduced Mn2+ effects. D, 3-fold increased adhesion was observed after treatment of Duffy-positive SRes with activating anti-β1 TS2/16 antibody (2 μg/ml) and 2 mM Mn2+ (***, p < 0.01; ***, p < 0.001; ***, p < 0.01). Pretreatment with the blocking anti-α4 HP2.1 (20 μg/ml) reduced Mn2+ effects. Columns and error bars represent means ± S.D. (Wilcoxon matched-paired test).

positive SRe adhesion to FN (Fig. 1B) and VCAM-1 (data not shown), indicating the involvement of α4β1 in this adhesion.

IL-8 and RANTES Exclusively Increase Duffy-positive SRe Adhesion—The activation of the α4β1/V CAM-1 and the α4β1/FN interactions was analyzed in Duffy-positive and Duffy-negative SRes after treatment by IL-8 or RANTES. Neither IL-8 nor RANTES was able to increase Duffy-negative SRe adhesion to FN or to VCAM-1 (Fig. 2A). Conversely, IL-8 increased Duffy-positive SRe adhesion to FN (11.50 ± 0.98%; p = 0.0005) and to VCAM-1 (12.50 ± 1.23%; p = 0.0039) compared with untreated cells (6.50 ± 1.08%; Fig. 2B). Similar results were observed with RANTES, with a significant increase of Duffy-positive SRe adhesion on FN (11.20 ± 1.12%; p = 0.0025) and on VCAM-1 (12.10 ± 1.43%; p = 0.0039) (Fig. 2B). Adhesion of IL-8- and RANTES-treated Duffy-positive SRes to FN and VCAM-1 was reduced by the blocking anti-α4 HP2.1 antibody (3.23 ± 0.60, 3.87 ± 0.25, respectively and 4.0 ± 0.26 and 3.8 ± 0.10, respectively) (data not shown), indicating that α4β1 integrin was activated by both chemokines in these cells.

Anti-β1 TS2/16 or Mn2+ Increases Both Duffy-negative and Duffy-positive SRe Adhesion—The question naturally arises as to whether divergent cations such as Mn2+ or activating anti-β1 antibodies, both known to induce a high affinity state in β1 integrins (18, 25), were able to stimulate Duffy-negative SRe adhesion on VCAM-1 or FN. Incubation of Duffy-negative SRes with anti-β1 TS2/16 antibody significantly enhanced adhesion on FN (18.0 ± 2.2%; p = 0.020) and VCAM-1 (17.8 ± 1.9%; p = 0.0038) compared with untreated cells (Fig. 2C). Similar results were obtained after incubation with Mn2+ (Fig. 2C) because 20.0 ± 3.9% of treated Duffy-negative SRes adhered to FN (p = 0.0078) and 20.0 ± 2.7% to VCAM-1 (p = 0.0038). Incubation of Duffy-positive SRes with either anti-β1 TS2/16 antibody or Mn2+ enhanced cell adhesion to FN (p = 0.0025 and p = 0.0005, respectively) and to VCAM-1 (p = 0.0039) (Fig. 2D). The blocking anti-α4 HP2.1 antibody reduced adhesion in both Mn2+-treated Duffy-negative (Fig. 2C) and Duffy-positive SRes (Fig. 2D).

Duffy-positive and Duffy-negative SRes Display Similar Erythroid α4β1 Expression but with Distinct Activation States—Increased adhesion of Duffy-positive SRes to FN and VCAM-1 could be attributable either to higher percentages of integrin α4β1-positive SRes, higher expression of α4β1 on the surface of these SRes or to conformational changes leading to higher proportions of activated erythroid α4β1 in these patients. To address these questions we analyzed SRes from Duffy-positive and Duffy-negative patients by flow cytometry. First, no significant differences were shown for the percentages of total circulating SRes (Fig. 3A) and for those of α4β1-positive SRes (Fig. 3B) between the two Duffy phenotype groups. The expression level of erythroid integrin α4β1 was estimated using a set of anti-α4 and anti-β1 antibodies. Expression was determined as a percentage of α4β1-positive reticulocytes and as α4β1-related mean fluorescence intensity (MFI). This analysis showed that α4β1-positive SRes from Duffy-negative (Fig. 3C) and Duffy-positive (Fig. 3D) patients expressed similar amounts of α4β1 integrin.
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FIGURE 4. α4β1 is differentially activated in Duffy-negative and Duffy-positive SRes. Flow cytometry analyses were assessed as described under “Experimental Procedures.” A, in Duffy-negative SRes, HUTS-21 mAb stained less SRes than MAR4, 4B4, and TS2/16 mAbs (**, p < 0.01) (Wilcoxon matched-paired test). B, in Duffy-positive SRes, HUTS-21, MAR4, 4B4, and TS2/16 mAbs stained similar percentages of SRes. C, in Duffy-negative SRes, HUTS-21-related MFI was close to the isotype-matched control antibody (data not shown) and was less than MFI of MAR4, 4B4, and TS2/16 mAbs (**, p < 0.01) (Wilcoxon matched-paired test). D, in Duffy-positive SRes, similar MFI values were obtained with HUTS-21, MAR4, 4B4, and TS2/16 mAbs. 5, HUTS-21 MFI in Duffy-negative SRe (C) was significantly different from that of Duffy-negative SRe (D) (**, p < 0.05) (Mann-Whitney test).

Next, activated forms of α4β1 on Duffy-negative and Duffy positive SRes were evaluated using the conformation-sensitive anti-β1 HUTS-21 antibody and three conformation-independent anti-β1 antibodies (MAR4, TS2/16, and 4B4) for detection of total α4β1. In Duffy-negative SRes (Fig. 4A), low binding of anti-β1 HUTS-21 was detected compared with anti-β1 MAR4 (p = 0.0039), anti-β1 4B4 (p = 0.0020), and anti-β1 TS2/16 (p = 0.0039). In Duffy-positive SRes (Fig. 4B), similar antibody binding levels were observed among the four anti-β1 antibodies, suggesting the preexistence of α4β1 under conformational activated states in these cells. The amounts of activated α4β1 were then evaluated by determining the HUTS-21-related MFI in both SRe populations as well as the MFI related to the other anti-β1 antibodies. This analysis showed less HUTS-21-related MFI in Duffy-negative SRes compared with MAR4, 4B4, and TS2/16 MFIs (Fig. 4C) (p = 0.0078) and, equivalent MFIs for all antibodies in Duffy-positive SRes (Fig. 4D). Note that HUTS-21 MFI in Duffy-negative SRes was similar to the MFI of the isotype-matched control antibody (data not shown). Importantly, the HUTS-21-related MFI of Duffy-negative SRes (Fig. 4C) was lower than that of Duffy-positive SRes (Fig. 4D) (p = 0.0039), indicating the presence of constitutively activated forms of α4β1 on Duffy-positive SRes.

IL-8 and RANTES Exclusively Induce α4β1 Clustering on Duffy-positive SRes—IL-8 increased Duffy-positive SRe adhesion to FN and VCAM-1 (Fig. 2B). We asked the question of whether it induces α4β1 conformational or avidity changes in these cells. First, Duffy-positive SRes were incubated or not with IL-8 and analyzed by flow cytometry using the conformation-specific anti β1 HUTS-21 antibody. Duffy-negative SRes were used as control. No differences were detected in the percentage of α4β1-positive reticulocytes in Duffy-positive (Fig. 5A) and Duffy-negative (Fig. 6A) after incubation with IL-8. Likewise, IL-8 had no effect on α4β1 expression level in both populations as determined by MFI (data not shown). Thus, IL-8 did not induce conformational changes of α4β1 in Duffy-positive SRes. Second, cell surface distribution of α4β1 on Duffy-positive and Duffy-negative SRes was probed with anti-α4 HP2.2 antibody using immunofluorescence staining. Interestingly, Duffy-positive SRes pre-treated with IL-8 (Fig. 5B) or RANTES (Fig. 5C) showed bright fluorescent α4β1 clusters compared with the diffuse staining of untreated cells (Fig. 5D). Inversely, IL-8 (Fig. 6B) and RANTES (Fig. 6C) had no effect on α4β1 distribution on Duffy-negative SRes as no clusters were observed on their surface compared with untreated cells (Fig. 6D). To characterize the expression of α4β1 integrin in Duffy-negative SRes further, immunofluorescence staining was performed using anti-α4 HP2.2 antibody in the absence or presence of MnCl2. As shown in Figs. 5E and 6E, MnCl2 was able to induce α4β1 clustering in both Duffy-positive and Duffy-negative SRes, respectively, indicating that the absence of α4β1 clustering in Duffy-negative SRes in response to IL-8 and RANTES was not due to intrinsic defects of α4β1 in these cells.

DISCUSSION

Sickle RBCs, namely the reticulocytes, express α4β1integrin that favors their adhesion to endothelium through different pathways, mainly α4β1/VCAM-1 and/or α4β1/FN pathways (2, 5), which are highly sensitive to IL-8 and RANTES stimulation (6, 7). Therefore, it remains an open question whether activation of α4β1 is coupled to DARC expression. Our adhesion experiments and fluorescence analyses performed either with activating or conformation-sensitive antibodies reveal differences in α4β1 affinity/avidity states between Duffy-negative SRes and Duffy-positive SRes. In the former group, the integrin was not constitutively active, whereas in the latter group it was in a constitutively intermediate active form. Thus, for the first time, a coupling between DARC expression and the activation process of α4β1 is shown.

The mechanisms involved in α4β1 activation (outside-in signaling) in SRes have been investigated by several groups (7, 8, 16–19, 26–28), but information related to DARC expression was lacking. When α4β1 activation on Duffy-negative SRes and Duffy-positive SRes was examined with respect to sensitivity to compounds known to interfere with integrin affinity and/or avidity, a differential behavior was noticed. In the case of Duffy-negative SRes, neither IL-8 nor RANTES was able to increase its basal adhesion (α4β1 activation). On the other hand, when experiments were performed in the presence of mAb β1 TS2/16 or MnCl2, adhesion of Duffy-negative SRes was increased by 6-fold on FN and VCAM-1, indicating that α4β1 adhesive properties could be strongly activated in these cells, suggesting that the absence of its activation by IL-8 and RANTES was due to a lack of out-
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![Diagram](image)

**FIGURE 5.** Chemokines induce $\alpha 4\beta 1$ clustering without affecting its conformation on Duffy-positive SRes. A, flow cytometry indicated that the percentage of $\alpha 4\beta 1$-positive SRes was not affected by IL-8. B–D, indirect immunofluorescence microscopy showing $\alpha 4\beta 1$ distribution after chemokine stimulation: cluster formation in Duffy-positive SRes induced by IL-8 (B) or RANTES (C) treatments compared with untreated cells (D). E, likewise, Mn$^{2+}$ was able to induce $\alpha 4\beta 1$ clustering on Duffy-positive SRes. Fluorescein (FITC) images were obtained on an inverted-phase fluorescence ECLIPSE 80i photomicroscope (Nikon). Scale bar, 10 $\mu m$.

Side-in signaling, most probably because of the absence of DARC in these cells. In the case of Duffy-positive SRes, the elevated adhesion already observed in basal conditions was significantly increased by IL-8 and RANTES but under the values observed when adhesion assays were performed in the presence of mAb TS2/16 or Mn$^{2+}$. These results are consistent with those of Kumar *et al.* (6), if one assumes that the patients enrolled in their study had predominantly the Duffy-positive phenotype. Indeed, these authors (6) have observed an increased adhesion of IL-8-treated sickle RBCs to human umbilical vein endothelial cells for 75% of the patients studied. The activation states of $\alpha 4\beta 1$ integrin which are primarily defined by their differential avidity to immobilized ligands are known to vary according to the cell type on which it is expressed (6–8, 16–19, 26–28). Based on these previous data and our results, we conclude that differences in adhesion between the two phenotypes could be due to distinct conformational states of $\alpha 4\beta 1$ molecules expressed in the two groups. Such a possibility is consistent with our fluorescence immunostaining experiments whose results are discussed below.

The population of cell surface integrins is in equilibrium between nonactivated form (low affinity) and an activated form (high affinity) (18, 19). Flow cytometry analyses demonstrated neither differences in the percentage of $\alpha 4\beta 1$-positive SRes nor in the amount of $\alpha 4\beta 1$ expressed in Duffy-negative and Duffy-positive patients. However, our results obtained with the conformation-sensitive HUTS-21 antibody indicated the absence of activated $\alpha 4\beta 1$ on the surface of Duffy-negative SRes compared with Duffy-positive SRes. Of particular significance also was the ability of $\alpha 4\beta 1$ to form clusters on the surface of Duffy-negative SRes after Mn$^{2+}$ stimulation, which is a characteristic of integrin activation. Thus, there was no deficiency in the ability of $\alpha 4\beta 1$ to be activated on Duffy-negative SRe, indicating that $\alpha 4\beta 1$ is expressed in a low affinity state on these cells that could shift toward a high affinity/avidity state after cell activation as observed for other cell types (8, 19). In Duffy-positive SRes, $\alpha 4\beta 1$ is expressed in an intermediate activated state which also shifted toward a high affinity state by TS2/16 antibody. However, IL-8 and RANTES did not affect affinity changes of $\alpha 4\beta 1$, whereas they increased Duffy-positive adhesion onto FN and VCAM-1, but with lower values than those observed with mAb TS2/16. Thus, the mechanism of $\alpha 4\beta 1$ activation by these chemokines might be distinct from that of TS2/16. Immunofluorescence staining showed that IL-8 or RANTES induced $\alpha 4\beta 1$ clustering before ligand binding as observed with phorbol esters (29).
However, affinity modulation and receptor clustering can play complementary roles in the adhesive function of Duffy-positive αβ1-positive SRes.

According to Pantaleo et al. (30), there is a linkage among proteins 4.1, band 3, and DARC on the red cell membrane which plays a key role in regulating membrane physical properties by stabilizing spectrin/actin interactions. In this regard, αβ1 conformation and therefore its activation states could be related to the presence or absence of DARC. More recently, we have also demonstrated that IL-8 and RANTES elicit a stimulation of the deoxygenation stimulated potassium loss via the Gardos channel in Duffy-positive but not in Duffy-negative sickle RBCs (31). Altogether, these data suggest a coupling between DARC and activation processes involved in increased adhesion and dehydration that play a role in the SCA pathogenesis.

Conflicting results have been reported regarding the clinical relevance of DARC erythroid expression in the pathophysiology of SCA (15, 30, 32, 33); Schnog et al. (32), and more recently Nebor et al. (15) failed to detect any association between DARC erythroid expression and the clinical severity of SCA. In contrast, Afenyi-Annan et al. (33) suggested an association between the lack of this receptor and kidney dysfunction. Based on these observations and on our results showing IL8/RANTES-induced αβ1 clustering on Duffy-positive SRes, we conclude that the presence of DARC, which modulates inflammatory processes, may determine the extent of inflammation in response to hypoxia in SCA.

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