

Adhesion of Sickle Neutrophils and Erythrocytes to Fibronectin

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The pathophysiology of vaso-occlusive crisis in sickle cell disease involves interactions among blood cells, plasma proteins, and vessel wall components. The initial goal of this work was to quantify the adhesion of sickle red blood cells (RBCs) to fibronectin immobilized on glass under both static and dynamic shear stress conditions. High-power microscopic inspection of static assay plates showed striking numbers of adherent neutrophils as well as RBCs. Sickle neutrophils and RBCs were significantly more adherent to fibronectin than the corresponding normal cells in static adhesion assays. Adhesion of both sickle neutrophils and sickle RBCs in dynamic adhesion assays was promoted by a period of static incubation preceding initiation of shear stress conditions. Adherent neutrophils remained attached at shear

stresses up to 51 dyne/cm²; most adherent RBCs were attached at shear stresses up to 13 dyne/cm², but detached at a shear stress of 20 dyne/cm². Sickle neutrophil adhesion was enhanced significantly by autologous plasma. Elevated levels of plasma interleukin-6 (IL-6; but not IL-1 or IL-8) were found in 6 of 9 sickle cell disease samples examined, and elevated levels of tumor necrosis factor were found in 2 of 9 samples. Plasma IL-6 levels correlated positively with both the number of sickle neutrophils adherent to fibronectin and the ability of sickle plasma to enhance adhesion of normal neutrophils to fibronectin. These data suggest possible roles for neutrophil activation and for fibronectin in mediating sickle neutrophil and RBC adhesion.

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MICROVASCULAR occlusion is responsible for much of the pathophysiology that underlies the clinical manifestations of sickle cell disease. Although vascular occlusion is mediated at least in part by the inability of poorly deformable, irreversibly sickled red blood cells (RBCs) to traverse the microcirculation, other vaso-occlusive processes have also been implicated. Sickle RBCs adhere abnormally to vascular endothelial cells (ECs), monocytes, and macrophages.¹⁻⁴ Studies using both static⁵⁻⁷ and dynamic⁸⁻¹⁰ experimental systems have shown that the abnormal adhesion of sickle RBCs to vascular ECs is modulated by autologous plasma factors.^{6,10-16} Plasma proteins that have been implicated in promoting sickle RBC adherence to vascular ECs include fibronectin,^{10,14,16} fibrinogen,^{7,13-16} high molecular weight von Willebrand factor multimers,^{10,17,18} and thrombospondin.¹⁹⁻²¹ In at least some cases, plasma proteins could serve as molecular bridges between integrins or integrin-like molecules, such as fibronectin receptors,²² on sickle RBCs and/or ECs.

Fibronectin (FN) is a large glycoprotein dimer^{23,24} that contains multiple binding sites for heparin,^{24,25} fibrin,²⁶ collagen,^{24,27} and cell surface integrins.²⁸⁻³⁰ Two forms of FN are biologically important. Cellular FN is synthesized and secreted by fibroblasts, ECs, and macrophages.³¹ Plasma FN is synthesized and secreted primarily by hepatocytes.^{32,33} FN expression is enhanced by epidermal growth factor,³⁴ transforming growth factor β ,^{35,36} and interferon- γ .^{37,38} Plasma FN is an acute-phase reactant.^{39,40} The increased plasma FN levels observed during inflammatory responses may be stimulated by stress hormones such as glucocorticoids⁴¹⁻⁴³ and by cytokines such as interleukin-6 (IL-6).⁴⁴⁻⁴⁶

Increasing evidence suggests that white blood cells (WBCs) may be involved in the initiation of vaso-occlusive crisis in sickle cell disease. Clinical studies show that elevated total WBC counts are common in sickle cell disease patients^{47,48} and that WBC counts greater than 15,000 cells/ μ L are associated with an increased risk of early death in sickle cell disease.⁴⁹ The severity of crisis has been found to correlate with the total WBC count in a population of greater than 1,000 patients.⁵⁰ Anti-inflammatory agents such as methylprednisolone may be effective in decreasing the duration of crisis episodes.⁵¹ Laboratory studies show that

WBCs are considerably less deformable than RBCs⁵² and that WBCs can affect microvascular perfusion by lodging in the capillary orifice and/or adhering to venous ECs.⁵³

The pathophysiology of microvascular occlusion in sickle cell disease is likely to involve multiple factors, including physical occlusion by sickle RBCs and/or WBCs, abnormal adherence between sickle RBCs and ECs, altered vascular reactivity, and others. We have initiated a series of investigations to study the adhesive properties of sickle RBCs, using as a model system purified plasma proteins immobilized on glass. The present study uses FN as an adhesion protein, because Burns et al⁹ have reported that sickle RBCs adhere as well to FN- as to EC-coated capillary tubes. Although we hypothesized initially that sickle RBCs adhere abnormally to FN, early observations showed large numbers of adherent WBCs (especially neutrophils) as well as RBCs under both static and dynamic shear stress conditions. The scope of this study therefore includes the adhesion of both sickle neutrophils and sickle RBCs to FN.

MATERIALS AND METHODS

Blood from 15 adult patients with homozygous SS sickle cell disease and 9 normal subjects (AA) was drawn into heparinized

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tubes. All patients with sickle cell disease were asymptomatic at the time of study. No patient was on hydroxyurea treatment. Blood samples were taken after informed consent was obtained according to protocols established by the Harvard Medical School for human subjects in medical research. Blood was centrifuged at 1,000g at 4°C for 10 minutes and the plasma was removed. Some plasma was used fresh in adhesion experiments and the remainder was stored at -70°C for cytokine analysis. The packed cells were resuspended in phosphate-buffered saline (PBS; 140 mmol/L NaCl, 15 mmol/L NaPO₄, pH 7.4), and WBCs were removed by passing the resuspended cells through cotton wool.⁵⁴ Passage through cotton wool was used rather than buffy coat removal to retain as many reticulocytes as possible.⁵⁴ The cotton wool wash was found to remove 39% ± 15% of WBCs present in the samples of whole sickle or normal blood. As determined by Wright's staining and manual counting of blood cells, the distribution of both sickle and normal WBCs, both before and after passage through cotton wool, was 60% to 65% neutrophils, 32% to 36% lymphocytes, and 2% to 3% monocytes. After the passage through cotton wool, cells were then washed twice with PBS and used immediately or stored overnight at 4°C in high potassium PBS (KPBS; 140 mmol/L KCl, 15 mmol/L NaPO₄, pH 7.4) supplemented with 10 mmol/L glucose. No difference in WBC or RBC adhesion was observed between sickle or normal samples tested immediately and samples tested after overnight storage.

Static adhesion assay. Glass coverslips (18 × 18 mm) were placed in 35-mm plastic Petri dishes and coated with various concentrations of FN (Sigma Chemical Co, St Louis, MO) in PBS (0.168 mL/coverslip) for 3 hours at 37°C.²² FN-coated coverslips and uncoated (control) coverslips were incubated overnight at 4°C, rinsed twice with PBS, and incubated with 1 mL of bovine serum albumin (BSA) at 20 mg/mL for 2 hours at 37°C. BSA coating was used to prevent nonspecific cell adhesion to the glass coverslips.²² Excess BSA solution was removed and coverslips were gently rinsed twice with PBS. Cells were washed and resuspended to 10% cell suspension in Hank's Balanced Salt Solution (HBSS) with 0.5% BSA, autologous plasma, or ABO- and Rh-compatible heterologous plasma. Protein-coated coverslips were incubated for 3 hours at 37°C with 1 mL of cell suspension. A 3-hour period of static incubation resulted in maximal WBC and RBC adhesion. Nonadherent cells were poured off and coverslips were rinsed 6 times with PBS. Adherent WBCs and RBCs were counted in 16 separate microscope fields using a microscope (CK2; Olympus, Lake Success, NY) at 630× magnification and an ocular grid. Cell counts were reported as the number of adherent cells per square millimeter. FN-specific adhesion was calculated as follows: (no. of cells per square millimeter adherent to FN-coated coverslip) - (no. of cells per square millimeter adherent to BSA-coated coverslip). Repeated measurements of WBC and RBC adhesion from the same SS patient on the same day yielded reproducible results. Measurements from the same patient on different days varied considerably, probably reflecting the heterogeneity of expression of sickle cell disease pathology.

Dynamic adhesion assay. A parallel plate flow chamber with a protein-coated slide as its base was used to investigate the adhesion of WBCs and RBCs to immobilized FN under well-defined fluid dynamic conditions.⁸ The flow chamber and glass slide were held in a parallel plate geometry by a vacuum maintained at the periphery of the slide. The height of the flow channel was controlled by the thickness of a silastic gasket through which the vacuum was transmitted. The chamber depth was machined to be 100 to 200 μm. Before assembly with the flow chamber, a 75 × 38 mm glass slide (Corning Glass Works, Corning, NY) was coated with 0.5 mL FN (50 μg/mL in PBS), incubated for 3 hours at 37°C, incubated overnight at 4°C, washed twice with PBS, coated with 8 mL BSA (20 μg/mL in PBS), and incubated for 2 hours at 37°C. Cells were

washed and resuspended to 1% cell suspension in M199 media (Fisher Scientific, Pittsburgh, PA). The cell suspension was maintained at 37°C and drawn by a syringe pump (Model 956; Harvard Apparatus, South Natick, MA) at a controlled flow rate to give a shear stress of 1 dyne/cm² in the flow chamber. Shear stress was calculated by using the momentum balance for a Newtonian fluid and assuming a parallel plate geometry and fully developed flow.⁸ The chamber was mounted on an inverted phase contrast microscope (Diaphot-TMD; Nikon, Garden City, NY) equipped with a CCD video camera (Model 72; Dage-MTI, Michigan City, IN). The microscope stage was maintained at 37°C by a thermostat controlled air stream incubator (Model ASI-400; Nicholson Precision Instruments, Gaithersburg, MD). All flow experiments were recorded in real-time on a 0.5-inch video cassette recorder (Model BV-1000; Mitsubishi, Cypress, CA) and displayed on a high-resolution monitor (Model PM-127; Ikegami, Maywood, NJ). A PC-based image processing system (Optimas; Bioscan, Edmunds, WA) was used to digitize video images for processing and analysis.

For each dynamic adhesion assay, the protein-coated slide was rinsed for 2 minutes with M199 media and then perfused for 5 minutes with the cell suspension. Nonadherent cells were removed by a 5-minute rinse with M199. Adherent WBCs and RBCs were counted in at least 10 fields and reported as the number of adherent cells per square millimeter. In some experiments, the rinse period was preceded by a 10-minute period during which cells were maintained in the chamber under static conditions (ie, in the absence of flow). FN-specific adhesion was calculated as follows: (no. of cells per square millimeter adherent to FN-coated slide) - (no. of cells per square millimeter adherent to BSA-coated slide).

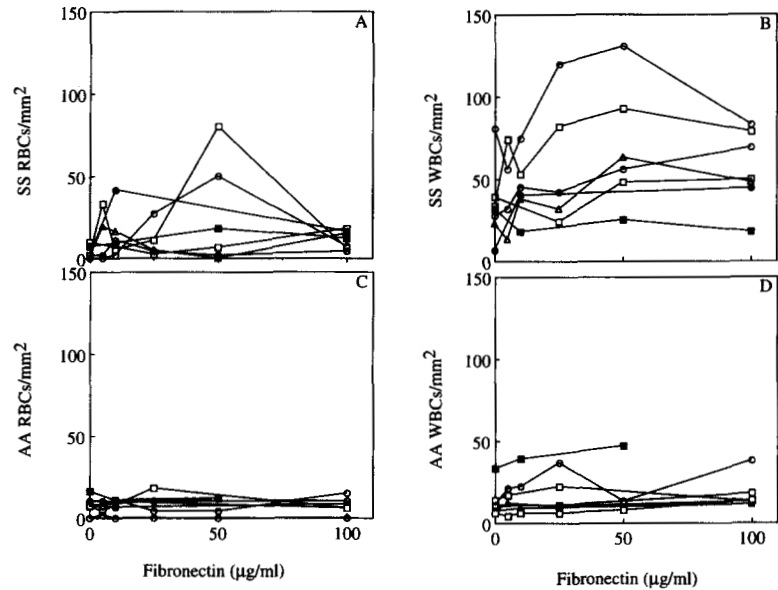
Detachment assays. The strength of WBC and RBC adhesion to protein-coated slides was quantified using detachment assays. The cell suspension was drawn through the parallel plate chamber for 5 minutes. Cells were then allowed to settle for 10 minutes under static incubation conditions. After the static incubation period, the chamber was perfused with M199 at an initial shear stress of 1 dyne/cm². By increasing the flow rate, the shear stress was increased every 2 minutes to a maximum of 51 dyne/cm². At the latter shear stress, all of the adherent RBCs (but few of the adherent WBCs) were removed. All experiments were recorded on videotape, and adherent WBCs and RBCs at the end of each 2-minute interval were counted and reported as the number of adherent cells per square millimeter.

Cytokine assays. The concentrations of the cytokines IL-1β, IL-6, IL-8, and tumor necrosis factor-α (TNF-α) in the plasma of sickle patients and normal controls were determined using ELISA Kits purchased from R & D Systems (Minneapolis, MN; IL-1β, IL-6, and IL-8) and Endogen (Cambridge, MA; TNF-α). Detection limits for these assay kits were as follows: IL-1β, 0.3 pg/mL; IL-6, 0.35 pg/mL; IL-8, 18.1 pg/mL; and TNF-α, 5 pg/mL. All analyses were performed in triplicate.

RESULTS

Sickle WBCs adhere specifically to fibronectin-coated glass under static incubation conditions. In 3-hour static adhesion assays, WBCs from patients with sickle cell disease were more adherent to both FN and BSA than were the corresponding normal WBCs (Fig 1 and Table 1). At 0 μg/mL FN (ie, in the presence of BSA alone), the adhesion of sickle WBCs to BSA was significantly greater than that of normal WBCs (Table 1). At 50 μg/mL FN, both the total and the FN-specific adhesion of sickle WBCs was significantly greater than that of normal WBCs (Table 1). Sickle WBC

Fig 1. Adhesion of sickle and normal RBCs and WBCs to FN under static incubation conditions. Coverslips were coated with various concentrations of FN, blocked with BSA (20 mg/mL), and incubated under static conditions with a cotton-washed 10% blood cell suspension. After 3 hours of incubation at 37°C, nonadherent cells were removed, coverslips were rinsed 6 times, and adherent cells were counted. Shown are adherent RBCs (A) and WBCs (B) from 5 different patients with sickle cell disease studied in 7 different experiments. Each symbol identifies a different patient. Note that two patients (denoted by \square and \circ , respectively) were each studied on 2 different days. Shown also are adherent RBCs (C) and WBCs (D) from normal individuals, performed in parallel (as indicated by matching symbols) to the experiments on blood from patients with sickle cell disease. Sickle RBC and WBC adhesion to FN was variable, but consistently greater than the adhesion of normal RBCs and WBCs.



adhesion to FN showed only a modest dependence on FN concentration, over the range 0 to 100 $\mu\text{g}/\text{mL}$ (Fig 1).

For any given patient with sickle cell disease, more WBCs than RBCs adhered to FN (Fig 1). This was an unexpected finding, because WBCs made up less than 1% of total blood cells, and the cotton wool wash⁵⁴ was designed to remove most of the WBCs from the cell suspension (see Materials and Methods). Although sickle blood cell suspensions contained 60% to 65% neutrophils, 32% to 36% lymphocytes, and 2% to 3% monocytes before incubation with FN, after static incubation with FN and rinsing of the coverslips, the distribution of adherent WBCs was 94% neutrophils and 6% lymphocytes and monocytes. Neutrophils were therefore the predominant sickle WBC type that adhered to FN-coated glass. The abnormal adhesion of sickle WBCs was not induced by the cotton wool washing procedure, because preparations of sickle blood from which most WBCs were removed by aspiration after centrifugation (ie, by buffy coat removal) showed numbers of adherent WBCs similar to those of sickle blood prepared using the cotton wool wash.

Sickle WBCs do not adhere specifically to fibronectin in the absence of prolonged static incubation. Experiments were designed to quantify the adhesion of sickle WBCs to FN both under continuous flow conditions at a shear stress of 1 dyne/cm² and under continuous flow conditions at a shear stress of 51 dyne/cm² after a 10-minute period of static incubation. Under continuous flow conditions at a shear stress of 1 dyne/cm², sickle WBCs were not significantly more adherent than normal WBCs to either FN or BSA. A 10-minute period of static incubation caused statistically insignificant increases in the numbers of adherent sickle and normal WBCs (Table 1). These data suggested that a prolonged period of static incubation was required for sickle WBCs to manifest specific adhesion to FN. Because it was difficult to count adherent WBCs in the presence of significant numbers of adherent RBCs, all WBC counts after the

static incubation period were performed after 12 minutes of continuous flow at shear stresses up to 51 dyne/cm². At the latter shear stress, all adherent RBCs were removed (see below). These observations suggested that sickle WBCs may be activated so that, allowed to settle during a prolonged period of static incubation, the cells adhered very tightly.

Autologous plasma enhances adhesion of sickle WBCs under static incubation conditions. Sickle RBC adhesion to ECs is enhanced in the presence of autologous plasma.^{6,10-16} We hypothesized that sickle WBC adhesion to FN was also enhanced by autologous plasma. Packed blood cells were prepared without cotton washing from patients with sickle cell disease and resuspended to 10% in autologous plasma or in plasma from ABO- and Rh-compatible normal individuals. The cell suspensions were then incubated for 3 hours at 37°C on coverslips coated with FN (50 $\mu\text{g}/\text{mL}$), the coverslips were rinsed, and adherent WBCs were counted. Under these static incubation conditions, the adhesion of sickle WBCs was enhanced in the presence of autologous plasma (Fig 2). The number of adherent sickle WBCs ranged from 10 to 777 cells/mm² in autologous plasma and from 10 to 223 cells/mm² in normal plasma. Blood samples from approximately half of the patients with sickle cell disease showed numbers of adherent WBCs in normal plasma that were greater than the maximum number of adherent control WBCs in normal plasma. For this subpopulation of patients, the degree of enhancement of WBC adhesion by autologous plasma was twofold to fourfold. In contrast, autologous plasma did not significantly increase the adhesion of sickle WBCs when the number of adherent WBCs in normal plasma was within the range of control values (Fig 2).

Plasma levels of IL-6 correlate with adhesion of sickle WBCs to fibronectin under static incubation conditions. The results presented in Fig 2 suggested that sickle plasma may contain factors that promoted the adhesion of sickle WBCs (especially neutrophils) to FN. Such factors could

Table 1. Fibronectin-Specific Adhesion of Sickle and Normal WBCs

Sample	Incubation Conditions	FN	Alb	FN-Alb
AA	Continuous flow	2 ± 1	1 ± 1	1 ± 0.3
AA	Static (10 min) then flow	5 ± 2	4 ± 2	1 ± 1
AA	Static (180 min)	16 ± 5*	13 ± 4†	3 ± 2‡
SS	Continuous flow	4 ± 1	5 ± 1	-1 ± 1
SS	Static (10 min) then flow	25 ± 10	21 ± 10	5 ± 4
SS	Static (180 min)	65 ± 14*	35 ± 9†	28 ± 8‡

Slides or coverslips were coated with FN (50 $\mu\text{g/mL}$) and blocked with BSA (20 mg/mL) (FN) or coated with BSA (20 mg/mL) alone (Alb). For continuous flow, cotton-washed blood cell suspensions (1%) were perfused over the protein-coated slides at room temperature for 5 minutes at 1 dyne/cm^2 . Immediately after perfusion, the slide was rinsed for 5 minutes with cell-free medium at 1 dyne/cm^2 . Adherent WBCs were then counted. For static (10 min) then flow, cotton-washed blood cell suspensions (1%) were perfused over the protein-coated slides at room temperature for 5 minutes at 1 dyne/cm^2 . Cells were then allowed to settle for 10 minutes under static incubation conditions. Cell-free medium was then perfused under increasing shear stress conditions, using staged increments of 2 minutes each. After the removal of all adherent RBCs at a shear stress of 51 dyne/cm^2 adherent WBCs were counted. For each experiment, 12 to 15 different fields were counted, and adherent cells were calculated as cells per square millimeter. FN and Alb values represent the mean \pm SEM for 7 different patients with sickle cell disease (SS) and 5 different controls (AA). For static (180 min), coverslips were incubated under static conditions with a cotton-washed 10% blood cell suspension. After 3 hours of incubation at 37°C, nonadherent cells were removed, coverslips were rinsed 6 times, and adherent cells were counted. FN and Alb values represent the mean \pm SEM for 5 different patients with sickle cell disease studied in 7 different experiments (SS) and 7 different controls (AA). Individual values for the static (180 min) condition are also shown in Fig 1. FN-Alb values represent the mean \pm SEM difference between WBC adhesion to FN and that to Alb for each individual sample (see the Materials and Methods). The significance of differences between results for identically treated SS and AA samples was tested by using the Student's unpaired two-tailed *t*-test (StatView 512+; Abacus Concepts, Berkeley, CA). Unless otherwise specified, $P > .05$. Prolonged static incubation was required for SS WBCs to manifest significantly increased specific adhesion to fibronectin as well as nonspecific adhesion to albumin.

* $P < .01$.

† $P < .05$.

‡ $P < .02$.

include the inflammatory cytokines IL-1, IL-6, IL-8, and TNF. Packed blood cells were prepared without cotton wool washing from 9 patients with sickle cell disease and from 5 normal controls and were resuspended to 10% in autologous plasma. The cell suspensions were then incubated for 3 hours at 37°C on coverslips coated with FN (50 $\mu\text{g/mL}$), the coverslips were rinsed, and adherent WBCs were counted. The concentrations of IL-1 β , IL-6, IL-8, and TNF- α were determined on matched samples of plasma. No IL-1 β was detected in plasma samples from either patients with sickle cell disease or normal individuals. Plasma IL-8 levels were 44 to 98 pg/mL in samples from both patients with sickle cell disease and normal individuals. TNF- α levels were 0 to 10 pg/mL in samples from normal individuals and in all but 2 samples from patients with sickle cell disease. The latter

2 samples had TNF- α levels of 34 pg/mL and 51 pg/mL and adherent WBC numbers of 390/ mm^2 and 770/ mm^2 , respectively. Thus, the 2 plasma samples with elevated TNF- α levels corresponded to 2 of the 3 blood samples that showed the greatest number of adherent sickle WBCs. IL-6 levels were 0 to 2.3 pg/mL in plasma samples from normal individuals and 1.3 to 6.7 pg/mL in samples from patients with sickle cell disease. There was a direct correlation ($R^2 = .74$, $P = .003$) between plasma IL-6 levels and the number of sickle WBCs adherent to FN in the presence of autologous plasma (Fig 3). In contrast, there was no correlation between the number of adherent sickle WBCs in autologous plasma and either the peripheral blood WBC count or the peripheral blood neutrophil count (data not shown). These results suggested that the inflammatory cytokines IL-6 and TNF- α were associated with increased adhesion of sickle WBCs to FN and that increased sickle WBC adhesion was not simply a function of the number of sickle WBCs or neutrophils in the sample.

Plasma levels of IL-6 correlate with enhancement of the adhesion of normal WBCs to fibronectin by plasma from patients with sickle cell disease. The results presented in Fig 3 suggested that plasma IL-6 levels could serve as a marker for factors (including IL-6 itself) that promote neutrophil adhesion to FN. To investigate this hypothesis, packed blood cells were prepared without cotton wool washing from normal individuals and were resuspended to 10% in autologous plasma or in ABO-compatible plasma from patients with sickle cell disease. The cell suspensions were then incubated for 3 hours at 37°C on coverslips coated with

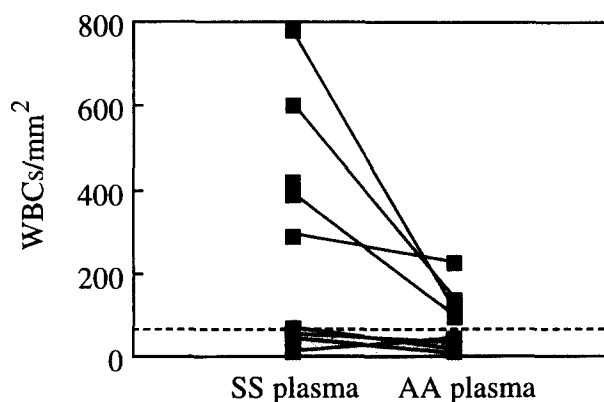


Fig 2. Enhancement by autologous plasma of sickle WBC adhesion to fibronectin. Whole blood from patients with sickle cell disease was centrifuged, and packed cells (WBCs and RBCs) were resuspended to a 10% cell suspension in autologous plasma (SS) or plasma from ABO- and Rh-compatible normal donors (AA). Coverslips were coated with FN (50 $\mu\text{g/mL}$), blocked with BSA (20 mg/mL), and incubated under static conditions with the cell suspension (1 mL). After 3 hours of incubation at 37°C, nonadherent cells were removed, each coverslip was rinsed, and adherent cells were counted. Very few RBCs remained attached. Solid lines connect data points obtained using blood cells from the same sickle patient. The dashed horizontal line represents maximum adhesion of control WBCs in autologous plasma. Sickle WBCs in autologous plasma were more adherent to FN than the same cells in normal plasma.

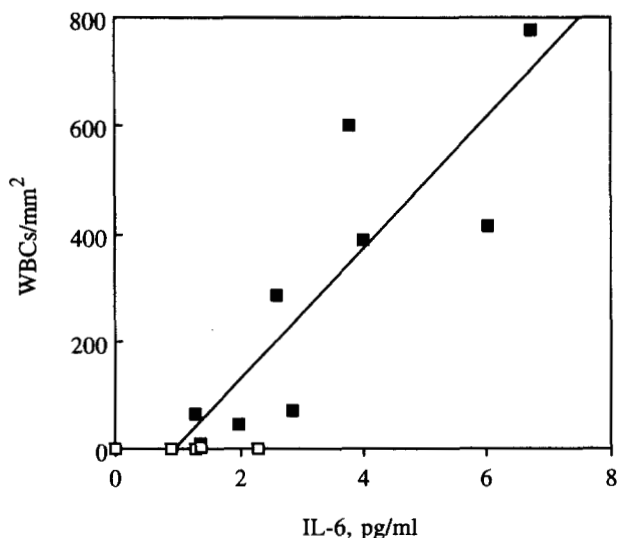


Fig 3. Correlation between plasma IL-6 levels and the adhesion to FN, under static incubation conditions, of sickle WBCs in autologous plasma. Sick and normal whole blood was centrifuged and packed cells (WBCs and RBCs) were resuspended in autologous plasma to make a 10% cell suspension. Coverslips were coated with FN (50 $\mu\text{g}/\text{mL}$), blocked with BSA (20 mg/mL), and incubated under static conditions with the cell suspension (1 mL). After 3 hours of incubation at 37°C, nonadherent cells were removed, each coverslip was rinsed, and adherent sickle (■) and normal (□) WBCs were counted. Very few RBCs remained attached. Sick and normal plasma was stored at -70°C, and IL-6 levels in thawed plasma were determined by ELISA. Plasma from 9 patients with sickle cell disease had IL-6 levels from 1.3 to 6.7 pg/mL and plasma from 5 normal controls had IL-6 levels from 0 to 2.3 pg/mL . There was a direct correlation ($R^2 = .74$, $P = .003$) between the number of adherent sickle WBCs and the level of IL-6 in autologous plasma.

FN (50 $\mu\text{g}/\text{mL}$), the coverslips were rinsed, and adherent WBCs were counted. The concentration of IL-6 was determined on matched samples of plasma. There was a direct correlation ($R^2 = 0.91$, $P = .0002$) between plasma IL-6 levels and the enhancement of the adhesion of normal WBCs to FN by plasma from patients with sickle cell disease (Fig 4). In contrast, there was no correlation between the enhancement of the adhesion of normal WBCs to FN by sickle plasma and either the peripheral blood WBC count or the peripheral blood neutrophil count of the SS patient from whom the plasma was obtained (data not shown). The inflammatory cytokine IL-6, or a plasma factor for which IL-6 serves as a marker, could therefore be responsible for neutrophil activation leading to enhanced adhesion to FN.

Sickle RBCs adhere specifically to fibronectin under static incubation and continuous flow conditions. In 3-hour static adhesion assays, RBCs from patients with sickle cell disease were more adherent to FN than were the corresponding normal RBCs (Fig 1). At 0 $\mu\text{g}/\text{mL}$ FN (ie, in the presence of BSA alone), the adhesion of sickle RBCs (3.7 ± 1.6 RBCs/ mm^2 , mean \pm SEM) was not significantly different from that of normal RBCs (8.0 ± 1.7 RBCs/ mm^2). At 50 $\mu\text{g}/\text{mL}$ FN, the FN-specific adhesion of sickle RBCs (29 ± 15 RBCs/ mm^2) was significantly greater ($P < .05$, Student's two-

tailed t -test) than that of normal RBCs (0 ± 2 RBCs/ mm^2). It is interesting that the adhesion of sickle RBCs to 50 $\mu\text{g}/\text{mL}$ FN was greater than that to 100 $\mu\text{g}/\text{mL}$ FN (Fig 1). Coating of high concentrations of FN on glass could lead to occupancy of lower-affinity FN binding sites on the glass surface. Transiently adherent sickle RBCs could then be released as the loosely bound FN desorbed from the glass surface during the adhesion assay.⁵⁵

Experiments were also designed to quantify the adhesion of sickle RBCs to FN (50 $\mu\text{g}/\text{mL}$) under continuous flow conditions at a shear stress of 1 dyne/ cm^2 . Both sickle and normal RBCs were significantly more adherent to FN than to BSA ($P < .04$, Student's two-tailed t -test). However, the specific adhesion of sickle RBCs to FN was not significantly greater than that of normal RBCs to FN under continuous flow conditions (Table 2).

Static incubation increases the adhesion of sickle RBCs to fibronectin. The results presented in Table 1 suggested that sickle WBC adhesion to FN was enhanced by a static incubation period preceding the initiation of continuous flow. To test the hypothesis that sickle RBC adhesion was also affected by incubation under static conditions, flow was interrupted for 10 minutes after perfusion of FN-coated slides for 5 minutes with a 1% suspension of cotton washed cells from sickle or normal blood in the parallel plate chamber.

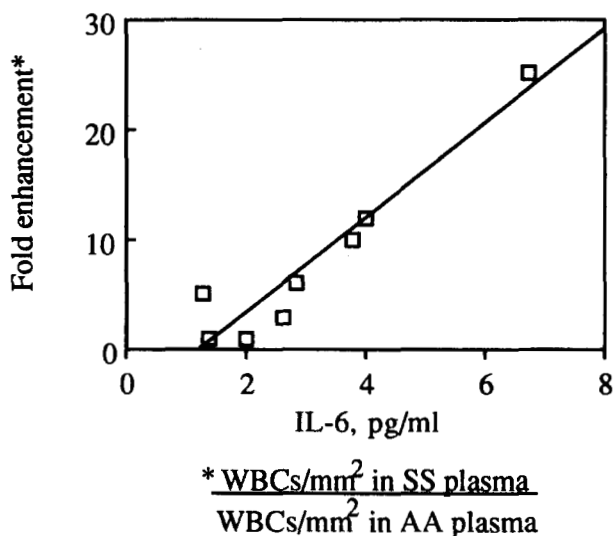


Fig 4. Correlation between sickle plasma IL-6 levels and the enhancement by sickle plasma of normal WBC adhesion to fibronectin. Normal whole blood was centrifuged, and packed cells (WBCs and RBCs) were resuspended to a 10% cell suspension in ABO, Rh-compatible plasma from patients with homozygous sickle cell disease. Coverslips were coated with FN (50 $\mu\text{g}/\text{mL}$), blocked with BSA (20 mg/mL), and incubated under static conditions with the cell suspension (1 mL). After 3 hours of incubation at 37°C, nonadherent cells were removed, each coverslip was rinsed, and adherent cells were counted. Very few RBCs remained attached. Plasma levels of IL-6 were determined as described in the legend to Fig 3. There was a direct correlation ($R^2 = .91$, $P = .0002$) between enhancement of normal WBC adhesion to FN (defined as adherent AA WBCs in matched sickle plasma/adherent AA WBCs in autologous AA plasma) and sickle plasma IL-6 levels.

Table 2. Fibronectin-Specific Adhesion of Sickie and Normal RBCs

Sample	Incubation Conditions	FN-Alb
AA	Continuous flow	16 ± 5*
AA	Static (10 min) then flow	920 ± 530†
SS	Continuous flow	38 ± 10*
SS	Static (10 min) then flow	6,610 ± 1,440†

Slides were either coated with FN (50 µg/mL) and blocked with BSA (20 mg/mL) (FN) or coated with BSA (20 mg/mL) alone (Alb). Cotton-washed blood cell suspensions (1%) were perfused over the protein-coated slides for 5 minutes at a shear stress of 1 dyne/cm², followed immediately by a 5-minute rinse with medium alone at 1 dyne/cm² (continuous flow) or by a 10-minute static incubation period followed by a 5-minute rinse with medium alone at 1 dyne/cm². Adherent RBCs were counted in 12 to 15 different microscope fields for each experiment and calculated as cells per square millimeter. Values represent the mean difference (±SEM) between the number of cells adherent to FN and the number of cells adherent to Alb for 8 different patients with sickle cell disease (SS) and 5 different controls (AA) (continuous flow) or for 9 different patients with sickle cell disease (SS) and 8 different controls (AA) (static then flow). The significance of differences between results for identically treated SS and AA samples was tested by using the Student's unpaired two-tailed *t*-test. Both SS and AA RBCs were significantly more adherent to FN than to BSA (*P* < .04, Student's two-tailed *t*-test). A significant difference between the number of specifically adherent SS RBCs and the number of specifically adherent AA RBCs was observed only after a 10-minute static incubation period.

* *P* > .05.

† *P* < .01.

After the period of static incubation, medium alone was perfused at a shear stress of 1 dyne/cm², and adherent RBCs were counted. The number of RBCs nonspecifically adherent to BSA-coated slides was subtracted from the number of RBCs adherent to FN-coated slides. The number of both sickle and normal RBCs adherent to FN was increased by a 10-minute period of static incubation preceding continuous flow conditions. For the 10-minute static incubation period (but not under continuous flow conditions; see above), the number of specifically adherent sickle RBCs was significantly greater than the number of specifically adherent normal RBCs (Table 2).

The strength of sickle RBC adhesion to fibronectin is less than that of sickle WBC adhesion. As noted above, after a period of static incubation, sickle WBCs remained adherent to FN at shear stresses up to 51 dyne/cm². To test the strength of adhesion of sickle and normal RBCs to FN, flow was interrupted for 10 minutes after perfusion of FN-coated slides for 5 minutes with a 1% suspension of cotton washed cells from sickle or normal blood in the parallel plate chamber. After the period of static incubation, medium alone was perfused at 2-minute staged increments of increasing shear stress, and adherent RBCs were counted at the end of each time interval. Under low shear stress conditions (1 dyne/cm²), the number of adherent sickle RBCs was threefold to fourfold greater than the number of adherent normal RBCs. A shear stress of 10 to 20 dyne/cm² was required to remove about half of the adherent sickle and normal RBCs, and all

adherent RBCs were removed by a shear stress of 51 dyne/cm² (Fig 5).

RBCs under high shear stress conditions were observed to deform before detachment from FN-coated slides. Cells frequently showed 2-point attachment to the substrate at shear stresses of 10 to 20 dyne/cm², and immediately before detachment cells uniformly exhibited 1-point attachment.

DISCUSSION

Vaso-occlusion in sickle cell disease is a multistep process⁵⁶ that involves initiation, propagation, and resolution phases.¹ Several potential mechanisms have been proposed for the initiation of vaso-occlusive events. First, adhesion of sickle RBCs to vascular ECs may initiate vaso-occlusion.⁵ Studies on individual sickle RBCs have shown that RBC adhesivity to ECs is most pronounced for the least dense fractions rich in reticulocytes and reversibly sickled RBCs and that adhesion is enhanced by autologous plasma, divalent cations, and collagen-binding plasma proteins.^{5,6,14,15} Dynamic adhesion studies on populations of density-fractionated sickle RBCs have also shown that the least-dense RBC populations are the most adherent to both cultured ECs^{8,18,57} and perfused animal microvascular beds.^{58,59} Dense, irreversibly sickled RBCs are unlikely to initiate vaso-occlusion, because such cells manifest low adhesivity to ECs in static

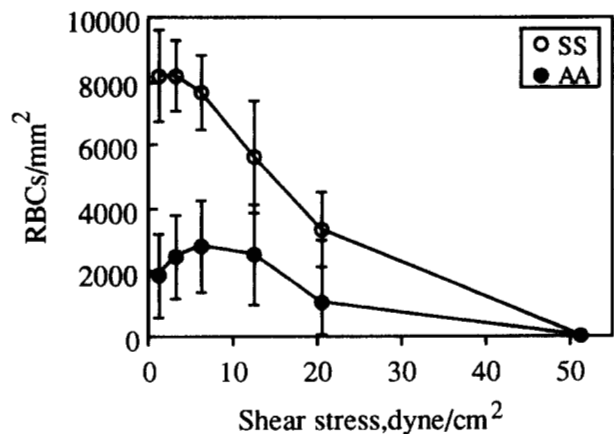


Fig 5. Fibronectin-mediated adhesion and detachment of sickle and normal RBCs. Slides were either coated with FN (50 µg/mL) and blocked with BSA (20 mg/mL) (FN), or coated with BSA (20 mg/mL) alone (Alb). Cotton-washed blood cell suspensions (1%) were perfused over the protein-coated slides for 5 minutes at 1 dyne/cm², followed by a 10-minute static incubation period. Medium alone was then perfused at 2-minute staged increments of increasing shear stress, and adherent RBCs in a single microscope field were counted at the end of each increment. Data points represent the mean (±SEM) difference between the number of cells adherent to FN and the number of cells adherent to Alb for 7 different patients with sickle cell disease and 6 different controls. FN supported adhesion of both sickle (SS) and normal (AA) RBCs after a 10-minute static incubation period, but the number of adherent sickle RBCs was threefold to fourfold greater than the number of adherent normal RBCs. A shear stress of 10 to 20 dyne/cm² was required to remove the majority of adherent sickle and normal RBCs, and all adherent RBCs were removed by a shear stress of 51 dyne/cm².

or dynamic adhesion models and there is no correlation between the frequency of clinical vaso-occlusive crisis and the percentage of dense cells.

Circulatory factors are also important in the initiation of microvascular occlusion. Sickle RBC adhesivity may be most relevant in low-flow, low-shear areas of the circulation (such as the postcapillary venules) and in areas in which vortex flow is found (such as vascular bifurcations or bends), because in these areas there is an increased probability of contact between circulating sickle RBCs and vascular ECs. Burns et al⁹ showed that the introduction of bends in either EC- or FN-coated capillary tubes promoted sickle RBC adherence. Other factors that may be involved in initiating vaso-occlusion include localized vasospasm or vasoconstriction, localized elevations in levels of plasma proteins induced by infection and/or dehydration,¹³ and endothelial abnormalities induced by viral infection⁶⁰ and cytokines.⁶¹

The propagation phase of vaso-occlusive crisis has been hypothesized to involve selective trapping of the densest and least deformable sickle RBCs in the microvasculature.⁶² Adhesion of sickle reticulocytes and reversibly sickled RBCs to postcapillary venules has been shown to facilitate the subsequent trapping of dense, irreversibly sickled RBCs, ultimately leading to microvascular obstruction.¹ The resolution phase of vaso-occlusion remains largely uncharacterized.

The most dramatic observation in this study is the extent to which WBCs (predominantly neutrophils) from patients with sickle cell disease adhere to FN-coated glass. Increased sickle WBC adhesion to FN is found under conditions of prolonged static incubation, and nonspecific sickle WBC adhesion under continuous flow conditions is enhanced by a period of static incubation. In the context of the model described above, increased WBC adhesion could contribute to both the initiation and propagation phases of microvascular occlusion in sickle cell disease. Enhanced sickle WBC adhesion could affect microvascular perfusion, initiate luminal obstruction, and potentially initiate vaso-occlusive crisis.⁵³ Furthermore, the enhancement of sickle WBC adhesion under static incubation conditions suggests a role for such adhesion in the propagation phase, after vascular flow is slowed by sickle RBCs (and/or WBCs) adhering to vascular ECs. One recent investigation reported that sickle WBCs are less adherent than control WBCs to glass coverslips perfused with whole blood in a dynamic adhesion assay.⁶³ Compared with control WBCs, sickle WBCs may be less adherent to human albumin, the major plasma protein coating the glass in the latter study,⁶³ and more adherent to FN (see the present study).

The increased adhesion of circulating WBCs from patients with sickle cell disease suggests that such cells may be activated. We hypothesize that sickle plasma contains a circulating WBC activator(s). In support of this hypothesis, we find that sickle WBC adhesion to FN is enhanced in autologous plasma. Furthermore, we show that sickle plasma increases the adhesion of normal WBCs to FN. Plasma-mediated increases in WBC adhesion to FN are observed despite the fact that the plasma concentration of FN is approximately

300 $\mu\text{g/mL}$. FN-specific adhesion could result under such circumstances because the local concentration of FN on the glass surface is likely to be many times this value. If the avidity (effective K_d) of plasma membrane FN receptors is greater than 300 $\mu\text{g/mL}$ but less than the local concentration of FN on the glass surface, then solution-phase FN in plasma would not be capable of displacing WBC binding to solid-phase FN. Alternatively, adhesive sites could be exposed on solid-phase FN that are not accessible on solution-phase FN. Circulating WBC activators include the cytokines IL-1, IL-6, IL-8, and TNF. IL-1⁶⁴ and TNF⁶⁵ are pleiotropic mediators of the inflammatory response. IL-6,⁶⁶ together with IL-1 and TNF, mediates the acute-phase response associated with inflammatory states. IL-6 also supports multilineage hematopoiesis by triggering the entry into cell cycle of multipotential stem cells in the bone marrow.^{67,68} IL-8⁶⁹ has proinflammatory activities including neutrophil chemotaxis and activation.

We report that plasma from patients with sickle cell disease contains higher levels of IL-6 than does control plasma. Elevated plasma levels of IL-6 have been reported in patients with increased numbers of circulating neutrophils,⁶⁹ B-cell dyscrasias,⁷⁰ solid tumors,⁷¹ and septic shock.^{72,73} Although the absolute levels of plasma IL-6 measured here in asymptomatic patients with sickle cell disease are lower than those found in severely ill patients with malignancy or septic shock, modest increases in IL-6 could nonetheless have significant biologic effects. IL-6 is synthesized by various cell types, including ECs, monocytes/macrophages, and T lymphocytes.⁶⁷ IL-6 is not produced constitutively, but the secretion of this cytokine is induced by viral infections, bacterial lipopolysaccharide, IL-1, and TNF.⁷⁴

Our results suggest that plasma IL-6 levels could serve as a marker for factors (including IL-6 itself) that promote neutrophil adhesion to FN. Human neutrophils express functional cell surface receptors for IL-6.⁷⁵ IL-6 treatment of neutrophils primes the respiratory burst^{76,77} and stimulates lysozyme secretion.⁷⁶ Furthermore, IL-6 induces increased synthesis of FN by monocytes and of FN and fibrinogen by hepatocytes.⁴⁴⁻⁴⁶ Human neutrophils also express cell surface receptors for both FN and fibrinogen.⁷⁸⁻⁸⁰ In patients with sickle cell disease, IL-6 could stimulate neutrophil (and RBC) production in the bone marrow and, in the periphery, both activate neutrophils and promote synthesis and release of adhesive proteins (especially FN and fibrinogen) that could serve as molecular bridges to promote sickle neutrophil adhesion to vascular endothelium or to subendothelial matrix proteins. In the absence of plasma, it is likely that the specific component of neutrophil binding to FN is mediated by FN receptors on the neutrophil surface. In the presence of plasma, neutrophil binding to FN could be mediated either directly, by cell surface FN receptors, or indirectly, by plasma proteins that serve as a molecular bridge between neutrophils and solid-phase FN. Plasma from patients with sickle cell disease could enhance the adhesion of normal neutrophils to FN by activation-associated increases in the number and/or avidity of cell surface FN receptors. IL-6 is one potential neutrophil activator that is present in greater

amounts in sickle than in normal plasma. Alternatively, sickle plasma could contain elevated levels of adhesive proteins (see above) and thereby enhance the adhesion of normal neutrophils to FN.

Our studies confirm the results of Burns et al⁹ showing that sickle RBCs adhere to FN-coated slides under continuous flow conditions. The adhesivity of sickle RBCs to FN is similar to that of sickle RBCs to other plasma proteins such as thrombospondin.^{19,21} When sickle RBCs are allowed to incubate on FN under static conditions, there is a large increase in the number of adherent RBCs. These data suggest that FN may play a role in mediating sickle RBC adhesion in the initiation and/or propagation phases of vaso-occlusive crisis. Others have also commented on the importance of slowing or stopping flow in enhancing the strength of interactions between RBCs and an adhesive surface.^{9,15}

Our data suggest an expanded model for vaso-occlusive crisis in sickle cell disease that involves RBCs, neutrophils, and plasma factors including FN and IL-6. Viral infections, bacterial infections, or other inflammatory states could increase plasma IL-6 levels and thereby increase the number of circulating neutrophils, enhance WBC adhesivity, and increase plasma FN levels. Under these conditions, activated WBCs could adhere more strongly to the surface of small blood vessels. Blood flow rates in these vessels would then be reduced and could be slowed to near-static conditions in some areas. The low or static blood flow induced by adherent neutrophils or RBCs, combined with the increased plasma FN stimulated by IL-6, could enhance further adhesion of sickle RBCs to ECs and to other previously adherent blood cells, and thus initiate and propagate vaso-occlusive crisis. Further experimental studies are needed to validate the individual steps of this expanded model.

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