

# Abnormal Adherence of Sick Erythrocytes to Cultured Vascular Endothelium

## POSSIBLE MECHANISM FOR MICROVASCULAR OCCLUSION IN SICKLE CELL DISEASE

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**ABSTRACT** The abnormal shape and poor deformability of the sickled erythrocyte (RBC) have generally been held responsible for the microvascular occlusions of sickle cell disease. However, there is no correlation between the clinical severity of this disease and the presence of sickled RBC. In searching for additional factors that might contribute to the pathophysiology of sickle cell disease, we have investigated the possibility that sickle RBC might be less than normally repulsive of the vascular endothelium. After RBC suspensions are allowed to settle onto plates of cultured human endothelial cells, normal RBC are completely removed by as few as six washes. In contrast, sickle RBC remain adherent despite multiple washes. On subconfluent culture plates, normal RBC are distributed randomly, whereas sickle RBC cluster around endothelial cells. Sickle RBC adherence is not enhanced by deoxygenation but does increase with increasing RBC density. The enzymatic removal of membrane sialic acid greatly diminishes the adherence of sickle RBC to endothelial cells, suggesting that sialic acid participates in this abnormal cell-cell interaction. Although net negative charge appears normal, sickle RBC manifest an abnormal clumping of negative surface charge as demonstrated by localization of cationized ferritin. These abnormalities are reproduced in normal RBC loaded

with nonechinocytogenic amounts of calcium. We conclude that sickle RBC adhere to vascular endothelial cells in vitro, perhaps caused by a calcium-induced aberration of membrane topography. This adherence may be a pathogenetic factor in the microvascular occlusions characteristic of sickle cell disease.

### INTRODUCTION

Perhaps the most prominent pathologic feature of sickle cell disease is the occurrence of microvascular occlusions, which are responsible for such clinical manifestations as painful crises and which have generally been attributed to the abnormal shape and poor deformability of the sickled erythrocyte (RBC).<sup>1</sup> However, there is no correlation between the clinical severity of sickle cell disease and the number of circulating irreversibly sickled cells (ISC) (1). Hence, we have sought factors in addition to abnormal shape and stiffness, which might contribute to abnormal microvascular blood flow in this disease.

Because the development of microvascular occlusions presumably involves an intimate contact between RBC and endothelium, we speculated that sickle RBC might be less than normally repulsive of endothelial cells. In this regard, it is interesting that sickle RBC have recently been reported to have a lower than normal membrane sialic acid (NANA) content (2). Because NANA is the major determinant of the RBC's negative surface charge (3), such a deficiency would provide a potential mechanism for an abnormal interaction be-

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<sup>1</sup>Abbreviations used in this paper: AR, adherence ratio; CF, cationized ferritin; DU, distance unit; HBSS, Hanks' balanced salt solution; Hct, hematocrit; ISC, irreversibly sickled cell(s); NANA, sialic acid; RBC, erythrocyte(s).

tween sickle RBC and the microvascular endothelium. Although the results of our investigations indicate that sickle RBC NANA deficiency is not the responsible mechanism, we nevertheless present evidence for an abnormal adhesiveness of sickle RBC to vascular endothelial cells. We suggest that this phenomenon may be of pathogenetic importance in the microvascular occlusive phenomena characteristic of sickle cell disease.

## METHODS

**Preparation of endothelial cells.** Endothelial cells obtained from human umbilical cord veins were grown to near-confluence in 35 mm plastic dishes (Falcon Labware Div. of Becton, Dickinson & Co., Oxnard, Calif.) as described (4). For experiments requiring more culture plates than can be derived from a single umbilical cord, endothelial cells harvested from several cords were pooled before culture to ensure that all plates used for a given experiment would be identical. Unless otherwise indicated, only plates of >90% confluence were used.

**Preparation of RBC.** RBC were obtained from normal individuals (homozygous for hemoglobin A) and from individuals with sickle cell disease (homozygous for hemoglobin S) or sickle trait (heterozygous for hemoglobin S). RBC from citrated whole blood were washed three times with isotonic NaCl; buffy coat was removed after each wash. RBC were then labeled for 40 min at 37°C with chromium-51 (sodium chromate; 50  $\mu$ Ci/ml packed RBC) to facilitate subsequent quantitation, washed an additional five times, and suspended to hematocrit (Hct) 25% in Hanks' balanced salt solution (HBSS) (Microbiological Associates, Walkersville, Md.) containing 0.5% human albumin (Cutter Laboratories, Inc., Berkeley, Calif.).

For some experiments, normal RBC were manipulated as follows before final suspension in HBSS/albumin. "High-dose" calcium loading was achieved by incubation (30 min at 37°C and Hct 10%) with 50  $\mu$ M ionophore A23187 in HBSS containing 100  $\mu$ M Ca (HBSS/Ca), followed by three washes with HBSS/Ca containing 3% albumin to remove ionophore (5); "low-dose" calcium loading employed 10  $\mu$ M ionophore and 10  $\mu$ M Ca (at Hct 50%). Metabolic depletion (95% ATP decrement) was induced by incubation (30 min at 37°C) with 10 mM NaF and 10 mM iodoacetamide in HBSS. NANA depletion was achieved by incubation of  $10^{10}$  RBC (at Hct 20% in phosphate-buffered saline) at 37°C with 0.25 U Sigma type IX *Clostridium perfringens* neuraminidase (Sigma Chemical Co., St. Louis, Mo.) for 5 min (20% depletion) or 50 min (95% depletion). The gross morphology of manipulated RBC was evaluated by light microscopic examination of wet mounts.

Subpopulations of RBC were obtained by centrifugation through a discontinuous density gradient of dextran-40 (Sigma Chemical Co.) as described by Abraham et al. (6). Certain of the resulting 7 *d* layers were selected: cells with *d* < 1.096 (g/ml) were designated "top layer"; cells with *d* > 1.096 and < 1.102 were designated "middle layer"; cells with *d* > 1.107 were designated "bottom layer." The middle layer contained <1% reticulocytes and no irreversibly sickled RBC. After density separation, RBC were washed six times with NaCl before final suspension in HBSS/albumin. To control for a possible membrane-altering effect of dextran, we performed control experiments using a nongradient centrifugation technique (7) and obtained similar results.

All experiments were done under ambient oxygen tension, except those comparing oxygenated and deoxygenated RBC. In the latter case, RBC were prepared by exposure to 100% N<sub>2</sub> in a tonometer and transferred to a N<sub>2</sub>-filled plastic glove bag, in which the adherence experiments were performed; control RBC were reoxygenated by exposure to room air.

**Endothelial adherence.** Endothelial cell culture plates were washed two times with HBSS/albumin, and 0.7 ml of each RBC suspension (Hct 25%) was layered on duplicate plates. After a 30-min static incubation at 37°C, plates were tilted and nonadherent RBC removed. Plates were then gently agitated 12 times with 1-ml wash volumes of HBSS/albumin, followed by three washes with distilled water to lyse residual adherent RBC. Each volume was removed by pipette and its radioactivity determined. The number of RBC remaining adherent after a given number of plate washes was then determined from the total counts per minute removed from the plate and the number of cells originally layered on. RBC counts were determined by Coulter counter (model ZB; Coulter Electronics Inc., Hialeah, Fla.).

**RBC NANA content.** Neuraminidase-susceptible NANA content was determined as described (8) using 0.25 U Sigma type IX neuraminidase per  $10^{10}$  RBC. The amount of enzymatically released NANA was determined by the Thiobarbituric acid method (9); blanks to correct for the presence of other chromogens had RBC incubated without enzyme.

**Incubation with cationized ferritin (CF).** Washed RBC were suspended to Hct 20% in HBSS and were prefixed with an equal volume of phosphate-buffered 2.5% formalin to avoid potential artifact caused by CF-induced clumping of charged surface groups (10). They were then suspended to Hct 10% in HBSS, 0.5 vol of CF (Sigma Chemical Co.) diluted 1:10 with isotonic NaCl was added, and the mixture was incubated at room temperature for 30 min (11). The RBC were then washed once with HBSS, suspended in HBSS, and an equal volume of 0.1% glutaraldehyde was added. Subsequent sample preparation and transmission electron microscopy were carried out as we have described (12). RBC from five normal individuals and five individuals with sickle cell disease were examined in this manner.

## RESULTS

**RBC adherence to cultured endothelium.** <sup>51</sup>Cr-labeled RBC from 17 individuals with sickle cell disease tended to remain adherent to confluent cultured endothelial cells despite repeated plate washing (Fig. 1A). Light microscopic examination of culture plates exposed to unlabeled RBC suspensions revealed identical results (data not shown) and demonstrated both ISC and RBC of normal morphology adhering to endothelial cells. Neither enrichment nor depletion of ISC was demonstrable among the adherent RBC. Approximately 1–10 sickle RBC remained adherent to each endothelial cell (at the end of 12 washes). That this represents the adherence of only a small percentage of the RBC in suspension was due to the vast excess of RBC ( $\sim 10^3$  RBC per endothelial cell) that were originally layered on the culture plates. When these experiments were repeated using the "nonadherent" RBC from previous plate washes, similar results were obtained. In addition, experiments using increasingly

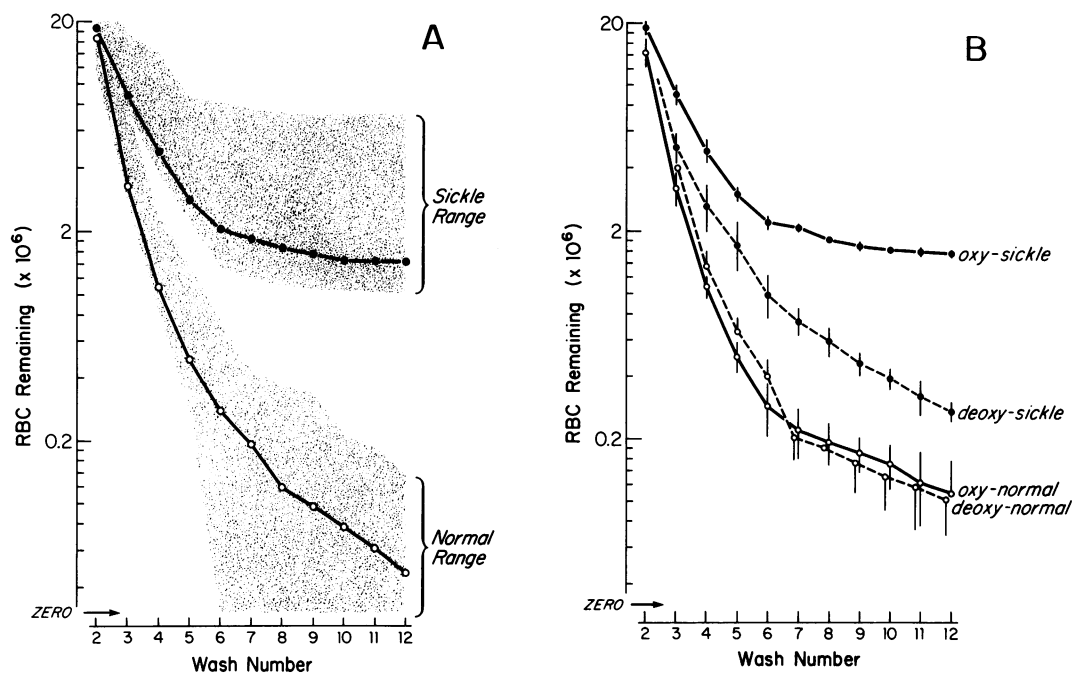


FIGURE 1 Sickie RBC adhere to confluent cultured endothelial cells. Adherence is expressed as the number of RBC remaining on culture plates after the number of plate washes indicated on the abscissa. (A) Sickie RBC tend to remain adherent despite multiple washes, while normal RBC continue to be removed (often completely by as few as six washes). Solid line within each range indicates the result (mean of duplicate plates) of a typical, single experiment. The corresponding curves for either normal or sickie RBC layered on control plastic plates (devoid of endothelial cells) are superimposable on the normal RBC curve (○). Each culture plate has  $\sim 1 \times 10^6$  endothelial cells. (B) Deoxygenation has no effect upon the removal of normal RBC from endothelial cells. However, the morphologic distortion of sickie RBC associated with deoxygenation diminishes their adherence. Vertical bars indicate the range for replicate plates in this representative experiment.

dilute RBC suspensions revealed that the percentage of sickle RBC adhering increased with decreasing Hct.

In contrast, normal RBC continued to be removed by successive plate washes and were removed as promptly as either normal or sickle RBC layered on control plastic plates devoid of endothelium (Fig. 1A). There was no difference between RBC from Caucasian normals, Negro normals, and individuals with sickle trait. Replacement of HBSS/albumin with human serum or citrated plasma (pooled type AB) for RBC suspension and plate washing did not alter these results.

Microscopic examination of subconfluent culture plates revealed that sickle RBC distribute themselves in rosette-like clusters around endothelial cells (Fig. 2A). Indeed, beyond the fifth plate wash,  $\sim 80\%$  of the sickle RBC were located directly adjacent to an endothelial cell. In contrast, the few remaining normal RBC were randomly distributed (Fig. 2B).

**Studies on manipulated RBC.** Because normal RBC were often completely removed by as few as six plate washes, an arbitrary adherence ratio (AR) was devised to facilitate comparison between sickle RBC and manipulated normal RBC. AR was defined as the

number of test RBC (sickle or manipulated normal RBC) remaining on culture plates after the fifth wash, divided by the number of normal RBC remaining on simultaneously handled plates. The mean adherence of replicate plates was used for this calculation. As indicated in Table I, the AR for the experiments contributing to Fig. 1A ranged from 1.59 to 17.60.

Sickle RBC adherence increased as RBC density increased, but even the least dense sickle RBC fraction manifested abnormal adherence to endothelium (Table I), indicating that aberrant shape per se is not required for adherence. Indeed, deoxygenation of sickle RBC actually decreased their adherence somewhat (Fig. 1B). Significantly, NANA depletion of sickle RBC dramatically decreased their adherence (Table I).

Various manipulations of normal RBC were employed in an attempt to induce adherence (Table I). 20% NANA depletion preserved normal RBC morphology and failed to induce adherence; even 95% NANA depletion delayed normal RBC removal only minimally. High-dose calcium loading (which increased mean corpuscular hemoglobin concentration by 40–55%) and metabolic depletion induced echino-

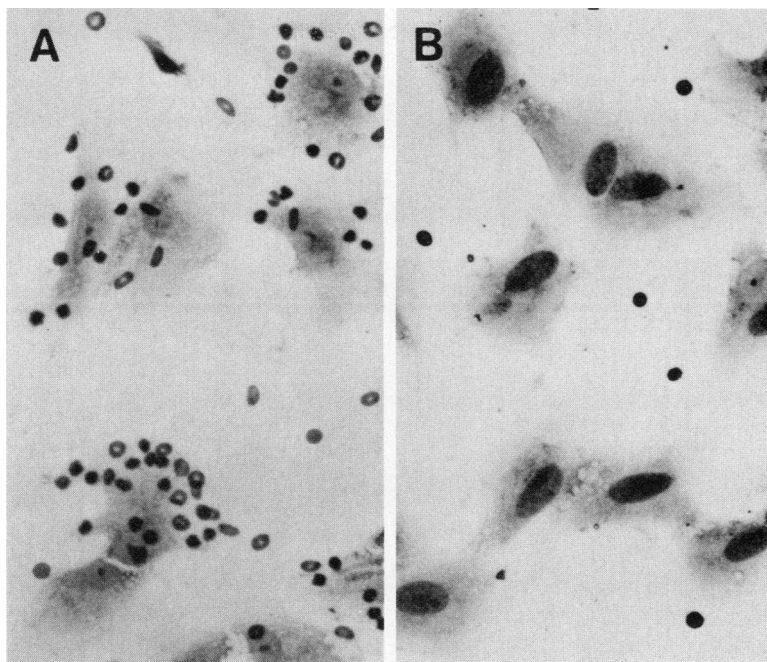


FIGURE 2 Distribution of RBC on subconfluent culture plates stained with Giemsa after the fifth plate wash. Sickie RBC distribute themselves in rosette-like clusters around endothelial cells (A), whereas normal RBC are present in fewer numbers and are randomly distributed (B).  $\times 375$ .

cytogenesis and failed to induce adherence. In contrast, low-dose calcium loading ( $10 \mu\text{M}$ ) preserved normal morphology, increased mean corpuscular hemoglobin concentration by only 5–8%, and did cause normal RBC to adhere to endothelium. NANA depletion of low-dose calcium-loaded normal RBC prevented adherence, as was the case with sickle RBC.

**RBC NANA content.** As others have reported (2), we found the membrane NANA content of sickle RBC to be lower than normal—but only if NANA was expressed as amount per milligram membrane protein (data not shown). If the same results were expressed as amount of NANA per RBC, we found no difference between sickle and normal RBC ( $14.96 \pm 0.79$  and  $14.74 \pm 0.59 \times 10^{-15}$  g NANA/RBC, respectively [mean  $\pm$  SE for seven individuals]).

**CF.** Upon incubation with CF, normal RBC acquired an homogeneous monolayer of ferritin particles (Fig. 3A and B). In striking contrast, RBC from individuals with sickle cell disease displayed an abnormal, clumped distribution of ferritin (Fig. 3C and D). A mathematical representation of this abnormal clumping is depicted in Fig. 4. In all sickle patient samples, at least 70% of the RBC showed the type of CF distribution designated “sickle”;  $>95\%$  of the RBC from all normal individuals revealed the CF distribution labeled “normal.”

Despite their preserved normal morphology, low-

dose ( $10 \mu\text{M}$ ) calcium-loaded normal RBC (which were abnormally adherent to endothelium) acquired the CF distribution seen in unmanipulated sickle RBC (Figs. 3 and 4).

## DISCUSSION

These data demonstrate that RBC from individuals with sickle cell disease adhere rather tenaciously to vascular endothelial cells in vitro. Although experiments on density fractionated RBC subpopulations suggest that at least some RBC from even the least dense sickle fraction are somewhat adherent, they clearly indicate that sickle RBC adherence increases with increasing cell density. Thus, we believe this phenomenon reflects a defect of the sickle RBC acquired during its in vivo aging (i.e., the accumulation of membrane damage [13, 14]).

Notwithstanding the fact that the most dense cell fraction is most adherent and contains most of the ISC, experiments on fully deoxygenated RBC suggest that adherence does not require the presence of frankly sickled RBC. Indeed, the increased distortion (reversible sickling) induced by deoxygenation appears to make sickle RBC less able to achieve a required degree of intimate contact with the endothelial cell. This difference between reversibly sickled RBC and ISC in propensity for adherence probably reflects the shape

TABLE I  
Adherence of RBC to Cultured Endothelial Cells,  
Expressed as an AR

Test RBC	AR*		
	Mean	±SD	Number of experiments
Normal individuals	1.00	0.36	†
Sickle cell disease patients (n = 17)	4.35§	3.20	17
Manipulated normal RBC			
Metabolic depletion	1.00	0.24	3
20% NANA depletion	1.32	0.46	3
95% NANA depletion	1.59	0.54	4
Calcium loaded (100 $\mu$ M)	1.05	0.31	4
Calcium loaded (10 $\mu$ M)	3.56	0.65	4
Calcium loaded (10 $\mu$ M) + NANA depletion	1.12	0.23	3
Manipulated sickle RBC			
95% NANA depletion	1.10	0.49	3
Density fractionated RBC			
Normal top layer	0.32	0.12	5
Normal middle layer	0.80	0.40	
Normal bottom layer	1.35	0.35	
Sickle top layer	1.56	0.47	
Sickle middle layer	3.50	1.47	
Sickle bottom layer	9.70	2.29	

\* AR is defined as the number of test RBC remaining adherent to endothelial cell culture plates after the fifth plate wash, divided by the number of normal RBC remaining on simultaneously handled plates. All experiments used duplicate plates; the mean adherence of the replicates was used for calculation of AR.

† This experiment examined the variability among seven different normal individuals. Hence, in this case, the result for each individual normal (test RBC) was divided by the mean for all normals.

§ Range, 1.59–17.60.

difference between the two cell types, the ISC being comparatively smooth (14, 15). It is perhaps analogous that calcium loading of normal RBC induces adherence only if nonechinocytogenic doses of calcium and ionophore are used.

Other manipulations chosen to simulate reported abnormalities of the sickle RBC failed to cause normal RBC to adhere to endothelial cells. For examples, 20% NANA depletion had no effect on normal RBC. Moreover, others have reported that the electrophoretic mobility of sickle RBC is normal (16), and we found no difference between normal and sickle RBC in NANA content (if expressed as amount per RBC). This suggests that the previously reported 19% deficiency in sickle RBC NANA (2) represents an artifact of data expression due to the increased amounts of extraneous protein in sickle RBC membranes (17).

Thus, a reduction in total RBC surface charge cannot

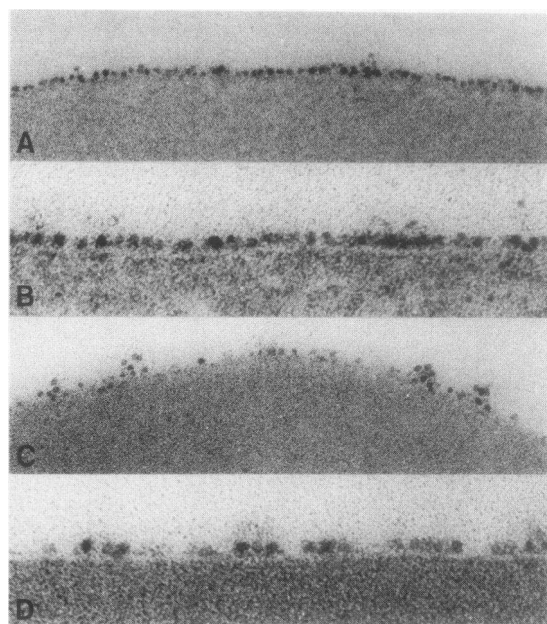


FIGURE 3 Electron microscopic localization of CF. Normal RBC (A and B) have an homogeneous monolayer of CF particles on their surface. In contrast, sickle RBC (C and D) have a patchy, interrupted, and clumped distribution of CF particles. Photographs are true cross-sections, the lower half of each panel being the inside of the RBC.  $\times 16,000$  (for A and C) and  $\times 26,000$  (for B and D).

explain the abnormal adherence of sickle RBC to endothelial cells. Nevertheless, the marked diminution in adherence manifested by NANA-depleted sickle RBC (and by NANA-depleted, calcium-loaded normal RBC) suggests that NANA somehow participates in this abnormal cell-cell interaction. Consequently, we speculated that sickle RBC might manifest local alterations in charge density. If this were true, an abnormal local clustering of negative charges (the majority of which are NANA residues [3]) would tend to leave reciprocal areas relatively lacking in negative charge. Either of these areas of abnormal local charge distribution might be available for interaction with apposite areas on the endothelial cell surface.

Our observations of the distribution of cationized ferritin are consistent with this hypothesis. The positively charged ferritin particles, which distribute themselves in correspondence with negatively charged groups on the RBC surface (11), are abnormally clustered on sickle RBC. Therefore, in view of the data indicating normal NANA content and electrophoretic mobility of sickle RBC, the abnormal surface distribution of CF indicates an aberration of charge distribution rather than charge quantity. That low-dose calcium loading of normal RBC causes them to adhere to endothelial cells and causes a similar abnormal distribution of CF suggests that this aberration of membrane topog-

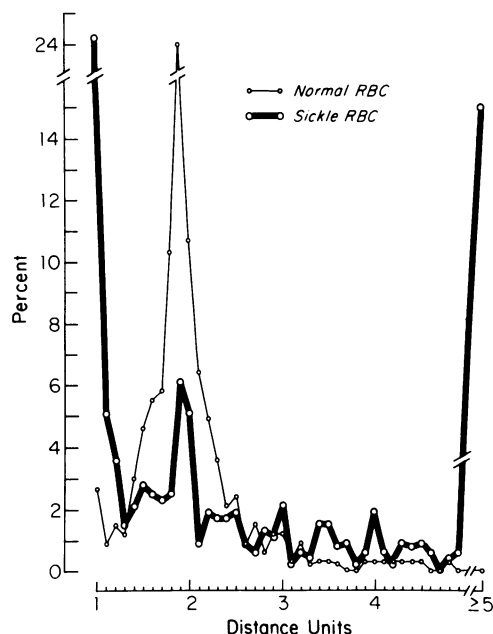


FIGURE 4 Frequency distribution of distances between adjacent CF. 1,000 interparticle distances were measured by examining high magnification ( $\times 180,000$ ) transmission electron micrographic cross-sections of RBC labeled with CF. The ordinate indicates the percentage of adjacent CF that have the interparticle distance indicated on the abscissa; distances were measured from particle center to particle center. Here, one distance unit (DU) is defined as one apparent CF diameter (since only the electron dense iron core of the CF particle is visualized by this technique). Hence, one DU represents only the apparent touching of adjacent CF on these sections of standard thickness. The greatest number of CF on sickle RBC (thick line) are either abnormally clumped (peak at 1.0 DU) or are separated abnormally ( $\geq 5.0$  DU). In contrast, the majority of CF on normal RBC (thin line) are regularly spaced (at 1.9 DU), with very few interparticle distances of 1.0 and none  $\geq 5.0$ .

raphy may underlie the abnormal adherence of sickle RBC to endothelial cells.

The mechanism by which this aberrant topography develops remains unknown. The sickle RBC membrane has been shown to have numerous abnormal characteristics (14). Among these is an increased amount of membrane-associated protein (17), most of which is probably hemoglobin or a precipitated derivative thereof (18). Because the sickle RBC membrane is presumably inherently normal until such defects are acquired, we may speculate that such a perturbation of the inner aspect of the RBC membrane leads subsequently to the other acquired abnormalities of the sickle RBC, such as abnormally high calcium content (19). These studies suggest that this increased RBC calcium (perhaps in conjunction with other cellular abnormalities) leads to topographical alterations of transmembrane proteins, one of which carries the bulk of RBC surface NANA (20).

We recognize that our investigations were carried out under conditions that are not strictly comparable to those in vivo. For example, cultured endothelial cells were used, and a static incubation preceded the more dynamic process of culture plate washing. However, we have also observed this abnormal adherence of sickle RBC to the endothelial cells of intact umbilical cord vessels filled with RBC suspensions and flushed continuously thereafter. We are currently attempting to develop a satisfactory dynamic (flowing) model to extend these in vitro observations.

The propensity for sickle RBC to adhere to endothelial cells is likely to increase their difficulty in traversing the microvasculature. This abnormal cell-cell interaction may play a pathogenetic role—perhaps even an initiating role—in the development of microvascular occlusions, such as those leading to painful crises in individuals with sickle cell disease.

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