Increased Adherence of Sickled and Phosphatidylserine-enriched Human Erythrocytes to Cultured Human Peripheral Blood Monocytes

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Abstract

The precise mechanism by which sickle erythrocytes (RBC) are removed from the circulation is controversial, although it is possible that enhanced recognition of these cells by circulating mononuclear phagocytes could contribute to this process. We investigated this possibility by interacting sickle cells with cultured human peripheral blood monocytes. Our results show that both irreversibly sickled cells (ISC) and deoxygenated reversibly sickled cells (RSC) had a higher avidity for adherence to monocytes than did oxygenated sickle and normal RBC. ISC were the most adherent cell type. Adherence of RSC to monocytes was found to be reversible; reoxygenation of deoxygenated RSC resulted in a significant decrease in RSC—monocyte adherence. Concomitant with alterations in sickle RBC adherence were alterations in the organization and bilayer distribution of membrane phospholipids in these cells. Specifically, enhanced adherence was associated with increased exposure of RBC membrane outer leaflet phosphatidylserine (PS) and phosphatidylethanolamine, whereas lack of adherence was associated with normal patterns of membrane phospholipid distribution. To investigate the possibility of whether the exposure of PS in the outer membrane leaflet of these cells might be responsible for their recognition by monocytes, the membranes of normal RBC were enriched with the fluorescent PS analogue 1-acyl-2[(*N*-4-nitro-benzo-2-oxa-1,3-diazole)aminocaproyl]-phosphatidylserine (NBD-PS) via transfer of the exogenous lipid from a population of donor phospholipid vesicles (liposomes). RBC enriched with NBD-PS exhibited enhanced adherence to monocytes, whereas adherence of RBC enriched with similar amounts of NBD-phosphatidylcholine (NBD-PC) was not increased. Furthermore, preincubation of monocytes with PS liposomes resulted in a ~60% inhibition of ISC adherence to monocytes, whereas no inhibition occurred when monocytes were preincubated with PC liposomes. These findings strongly suggest that erythrocyte surface PS may be a ligand recognized by receptors on human peripheral blood monocytes and that abnormal exposure of PS in the outer leaflet of the RBC membrane, as found in sickle RBC, might serve to trigger their recognition by circulating monocytes. Our results further suggest that abnormalities in the organization of erythrocyte membrane phospholipids may have significant pathophysiologic implications, possibly including shortened cell survival.

Introduction

Although the average lifespan of circulating sickle erythrocytes (RBC)[1] is considerably shorter compared with that of normal RBC (1), the precise mechanism by which this pathologic cell is removed from the circulation is unknown. It has been suggested that a component underlying decreased sickle cell survival is the decreased deformability of these cells (2), resulting in an increased mechanical fragility of the sickle RBC. However, recent demonstrations that sickle RBC are unusually adherent to cultured endothelial cells (3, 4), artificial lipid vesicles (5), and macrophages (6) suggest that sickle RBC may possess a membrane abnormality which increases their propensity for intermembrane interactions. In this context, it is noteworthy that Tanaka and Schroit (7) have recently shown that the insertion of exogenously supplied analogs of phosphatidylserine (PS) into mouse RBC membranes stimulated their adherence to and phagocytosis by cultured mouse peritoneal macrophages, whereas similar insertion of an analogue of phosphatidylcholine (PC) was without effect. It was suggested by these authors that PS in the outer leaflet of mouse RBC might serve as a ligand which could be recognized by specific PS receptors on murine macrophages.

It is well known that, in normal RBC, PS is found exclusively in the inner leaflet of the RBC plasma membrane (8). However, in irreversibly sickled RBC (ISC) or deoxygenated reversibly sickled RBC (RSC), PS is found in both the inner and outer membrane leaflets (9). These observations have led us to investigate the relationship between the abnormal exposure of outer leaftlet PS in sickle RBC and their adherence to cultured human peripheral blood monocytes as a possible explanation for the increased hemolytic component (i.e., decreased cell survival) associated with sickle cell disease.

Methods

Materials

1-acyl-2[(*N*-4-nitro-benzo-2-oxa-1,3-diazole)aminocaproyl]-phosphatidylcholine (NBD-PC) was purchased from Avanti Polar Lipids,

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1. *Abbreviations used in this paper:* AR, adherence ratio; DQ, dequenching; ISC, irreversibly sickled cell; NBD-PC, 1-acyl-2[(*N*-4-nitro-benzo-2-oxa-1,3-diazole)aminocaproyl]-phosphatidylcholine; NBD-PS, NBD-phosphatidylserine, PC, phosphatidylcholine; PS, phosphatidylserine; RBC, erythrocyte; RSC, reversibly sickled cell; SUV, small unilamellar vesicle; WBC, leukocyte.

Inc. (Birmingham, AL). 1-acyl-2[*N*-4-nitro-benzo-2-oxa-1,3-diazole)-aminocaproyl]-phosphatidylserine) (NBD-PS) was prepared from NBD-PC by phospholipase D-catalyzed base exchange in the presence of L-serine and purified as described previously (7). Analysis of the product by thin-layer chromatography in basic, acidic, and neutral systems revealed a single fluorescent phosphate- and ninhydrin-positive spot. Stractan II was purchased from St. Regis Paper Co. (Tacoma, WA) and prepared as described previously (5). Hanks' balanced salt solution (HBSS), Earle's balanced salt solution, Eagle's minimal essential medium, and Roswell Park Memorial Institute cell culture medium 1640 (RPMI 1640) were obtained from Grand Island Biological Co. (Grand Island, NY). Percoll and Ficoll were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade from standard sources.

Procedures

RBC preparation. After obtaining informed consent, fresh blood samples from healthy normal individuals (normal hemoglobin, Hb AA) or patients with sickle cell disease (homozygous sickle hemoglobin, Hb SS) were collected in sodium heparin. Cells were separated from plasma by centrifugation (at 700 *g* for 5 min at 4°C), leukocytes (WBC) were removed by aspiration of the buffy coat, and the resulting RBC suspension was washed three times with phosphate-buffered saline (PBS) and resuspended to 20% hematocrit in PBS. Cell counts (RBC and WBC) were obtained using the Coulter model S electronic cell counter (Coulter Electronics Inc., Hialeah, FL). WBC contamination generally represented <500 cells/μl of RBC suspension and was similar for both normal and sickle RBC preparations. To quantify RBC uptake by monocytes, ^{51}Cr-labeled RBC from normal and sickle cell patients were used. The cells were labeled by the addition of ~200 μCi of $Na_2[^{51}Cr]O_4$ to 2×10^9 RBC for 1 h at 37°C. The RBC were then washed and subsequently employed in the experimental procedures detailed below. Deoxygenated RBC were prepared by incubating the RBC in a stoppered flask that was continuously flushed with humidified nitrogen for 1 h at 37°C. Oxygenated RBC were incubated in room air. Samples of RBC after oxygenation or deoxygenation were fixed in PBS containing 2% (final concentration) paraformaldehyde in which oxygenated or deoxygenated conditions were maintained.

Preparation of cohort RBC populations. Blood (20–40 ml) from normal and sickle cell patients was collected in heparinized tubes, RBC were separated from WBC and plasma by centrifugation (100 *g* for 5 min at 4°C), and the RBC were further washed three times in 15 vol of PBS containing 5 mM potassium and 11 mM glucose. To prepare cohort RBC populations, RBC were washed and resuspended to 20% hematocrit in PBS containing 5 mM potassium and 11 mM glucose and layered onto a discontinuous gradient of Stractan II, prepared as described previously (5). Gradients were centrifuged for 30 min at 4°C in a Beckman SW 25.1 rotor (Beckman Instruments, Inc., Fullerton, CA) at 20,000 *g*. RBC from the top (21–22% Stractan), middle (23–24% Stractan), and bottom (25–27% Stractan) fractions were collected, washed three times with PBS, and resuspended to 20% hematocrit in PBS. Top fractions of normal and sickle RBC contained <8% and >25% reticulocytes, respectively, as determined by methylene blue-stained smears. Middle and bottom fractions of both normal and sickle RBC contained <2% reticulocytes. Bottom fractions of sickle RBC contained ~50% ISC.

Isolation and culture of human monocytes from mononuclear blood leukocytes. Mononuclear blood leukocytes were collected from the peripheral blood of normal donors by separation on lymphocyte separation medium (Litton Bionetics, Kensington, MD) and washed twice in HBSS. Peripheral blood monocytes were isolated from the leukocytes by further separation on a preformed continuous Percoll gradient (10). Briefly, the leukocytes (4×10^7) were layered onto preformed Percoll gradients in 15-ml polycarbonate tubes and spun in swing-out buckets in a refrigerated centrifuge at 1,000 *g* for 20 min. Upon centrifugation, cell populations layered on top of the Percoll gradient separated on the basis of their relative densities into two distinct bands. The upper band was enriched in monocytes (80–90%) as determined by nonspecific esterase staining and morphologic examination. The cells from this band were harvested, washed twice in HBSS, and then resuspended in RPMI 1640 with 5% heat-inactivated human AB serum. After esterase staining, the suspension was adjusted to contain 1×10^6 monocytes/ml. Monocytes (1×10^5) were added to each well of a 96-well flat-bottomed Microtest II plate (Falcon Plastics, Oxnard, CA) that had been pretreated with fetal bovine serum for 1 h at 37°C. After incubation, the nonadherent cells were removed by washing with medium and the plates were washed three times with RPMI 1640. The plating efficiency of the monocytes was ~90%. The purity of monocytes at this point was >99%, as assessed by the ability of the cells to ingest carbon particles, by examination of the cell morphology, and by uptake of nonspecific esterase staining by the cells. Moreover, practically all of the adherent cells stained positively with the monoclonal antibody 61D3 directed against human monocytes (Bethesda Research Laboratories, Gaithersburg, MD).

Preparation of vesicles. Small unilamellar vesicles (SUV) were prepared from NBD-PC (mol wt 757) or NBD-PS (mol wt 741) (10 μg lipid/ml PBS) by sonication for 1 h at 10°C in a bath-type sonicator under nitrogen. Contaminating multilamellar vesicles were removed (pelleted) from the SUV suspension by centrifugation at 100,000 *g* for 1 h at 10°C.

Vesicle-RBC incubations. Washed ^{51}Cr-labeled RBC (10^7 RBC/ml) were incubated with the indicated SUV suspensions for 30 min at 37°C. RBC were collected by centrifugation (1,000 *g* for 5 min at 22°C) and then washed three times with 15 vol of warm PBS. The washed RBC were then resuspended to 10^7 RBC/ml in PBS.

To quantitate the amount of NBD lipid transferred from the SUV to the RBC, aliquots of the treated RBC (10^7 cells) in 1 ml of PBS were lysed by the addition of 0.1 vol of 10% sodium dodecyl sulfate (SDS) (1% final concentration). The relative fluorescence was then compared to a standard curve of relative NBD fluorescence at 525 nm (λex 470 nm) with known amounts of NBD-PS or NBD-PC in 1 ml of PBS containing 10^7 RBC and 1% SDS. The increase in fluorescence was linear in the range of interest (0–500 ng of NBD-phospholipid/ml).

Fluorescence measurements. Steady-state emission spectra were obtained by using an Aminco SPF-500 fluorescence spectrophotofluorometer (Aminco, American Instrument Co., Silver Spring, MD). All samples were excited at 470 nm and emission was recorded at 525 nm using narrow band-pass slits to minimize scatter effects. Confirmation of lipid transfer from the population of donor vesicles to the RBC was obtained using an assay based on lipid dequenching (DQ) upon transfer and subsequent dilution of fluorescent lipid analogues into the cell membranes, as previously described (7). The extent of DQ in a sample was calculated from the relative intensities of the NBD-labeled lipids in washed vesicle-treated RBC in the absence and presence of detergent (Fc) compared with the ratio of fluorescence of the initial vesicle population in the absence and presence of detergent (Fv) by the following relationship:

$$DQ = (Fc - Fv)/(1 - Fv) \times 100. \quad (1)$$

Fc and Fv were corrected for sample dilution by the addition of detergent and the differences in quantum yield of the fluorophore in the various assay systems, as described previously (7).

Monocyte–RBC adherence assay. Aliquots of ^{51}Cr-labeled RBC from normal and sickle cell patients (0.2 ml, 5×10^7 RBC/ml of PBS) were added to wells containing the cultured human monocytes (RBC to monocyte ratio of 100:1) and incubated for 1 h at 37°C in a humidified CO_2 incubator. The cells were then washed three times with PBS to remove unbound RBC and the remaining adherent cells were lysed by the addition of 0.1 ml of 0.1 N sodium hydroxide. The lysate was then monitored for ^{51}Cr radiation using a gamma radiation spectrometer. In those experiments where deoxygenated RBC were used, the RBC-monocyte incubation and subsequent cell washings were performed in a sealed chamber constantly flushed with humidified nitrogen. In some experiments, results are presented as a RBC–monocyte adherence ratio (AR), that is, the ratio of the percentage of

deoxygenated/oxygenated RBC adhering to monocytes or the percentage of sickle/normal RBC adhering to monocytes. It should be noted that monocytes are capable of ingesting adherent cells; indeed, phagocytosis normally proceeds through an adherence → endocytosis process. However, the latter event requires the involvement of monocyte oxidative glycolysis. Because some of our experiments were conducted in anoxic environments, only erythrocyte adherence to monocytes under these conditions would occur. Thus, to be consistent with terminology throughout this paper we have referred to monocyte–erythrocyte uptake as representing monocyte–erythrocyte adherence, although the potential for erythrophagocytosis cannot be ruled out in those experiments conducted in oxygen-adequate environments.

Microscopy. RBC–monocyte adherence assays were performed as described above except the monocytes were grown on 12-mm glass coverslips placed in 15-mm diameter 24-well culture plates. After appropriate incubation times, the cells were fixed by the addition of 2% paraformaldehyde and observed by Zeiss Nomarski differential interference-contrast microscopy (Carl Zeiss, Inc., Thornwood, NY). In some instances, the specimens were further processed for scanning electron microscopy, as described previously (11). Photomicrographs were taken of selected fields to show the morphological characteristics of the adherence phenomenon.

Results

Adherence studies. ^{51}Cr-labeled normal and sickle RBC separated into cohort populations based upon density by centrifugation through discontinuous Stractan gradients were incubated with cultured normal human monocytes at 37°C. As shown in Table I, sickle RBC from the bottom (most dense, ISC-enriched) fraction were more adherent to monocytes than were the other RBC populations. In an effort to determine whether this observation indeed represented preferential recognition of ISC in the "dense" Stractan fraction, ISC were admixed at different ratios with the ISC-poor "middle" Stractan fraction and incubated for 1 h at 37°C with the monocytes. The results presented in Table II show a concomitant enhancement in RBC uptake with increasing ISC fractional content up to ~20–30% ISC. Further increases in the fractional content of ISC resulted in additional monocyte adherence although the extent of the increases were less dramatic. The reason for this phenomenon is not known but may represent saturation of the monocyte surface moiety responsible for ISC binding (see Discussion).

Table I. Adherence of Density-separated RBC to Cultured Peripheral Blood Monocytes

Stractan fraction (%)	% Adherence: Sickle RBC	% Adherence: Normal RBC	AR: Sickle/normal	% ISC
	%	%		%
Mixed RBC population	0.4±0.05	0.4±0.20	1.0 (1.4–0.8)	10
Top (21–22%)	0.4±0.1	0.4±0.05	1.0 (1.2–0.8)	1
Middle (23–24%)	0.6±0.1	0.5±0.20	1.2 (1.6–0.9)	4
Bottom (25–27%)	1.3±0.2	0.5±0.05	2.6 (3.3–1.9)	44

Monocytes isolated from fresh human peripheral blood by Ficoll and Percoll sedimentation were cultured overnight at 37°C. ^{51}Cr-labeled normal or sickle RBC (RBC to monocytes = 100:1), either as mixed RBC populations or as cohort populations derived by Stractan-density centrifugation, were then incubated with the monocytes for 1 h at 37°C. The plates were then washed three times with PBS and the amount of adherent RBC (mean±SD) was determined as described in Methods. Results are presented as an adherence ratio (AR): sickle RBC adherence/normal RBC adherence, and represent the mean of five determinations derived from three individual patient samples. Ranges for AR values are given in the parentheses. Percentage ISC was determined by morphologic examination of paraformaldehyde-fixed RBC.

Table II. Correlation of Sickle RBC–Monocyte Adherence with the Fractional Content of ISC

% ISC	RBC adherence
%	*% of control*
0	100
10	108±3
20	138±2
30	142±6
40	146±4
50	153±3

Preparation of monocytes and ISC and adherence assays were performed as described in Table I. RBC fractions containing <50% ISC were obtained by mixing with RSC (middle Stractan fractions) from the same donor. Control cells (0% ISC) were normal RBC. ISC adherence to monocytes is expressed as the percentage increase in adherence of ISC-containing fractions over that of normal RBC. Results are the mean±SD of seven determinations derived from three individual patient samples.

Microscopic examination of fixed monocyte-RBC mixtures revealed the presence of ISC-monocyte rosettes (Fig. 1) and confirmed the observations shown in Tables I and II. Normal RBC and sickle RBC from the top and middle Stractan fractions were essentially incapable of forming such rosettes. Taken together, these results strongly suggest that ISC contain some cell surface component(s) that enhance their recognition by human monocytes, as evidenced by their avid adherence to these cells.

We next examined the adherence of oxygenated and deoxygenated normal and sickle RBC to monocytes. As shown in Table III, deoxygenating normal RBC had little effect on their adherence to monocytes. In contrast, deoxygenating sickle RBC induced dramatic increases in the extent of their adherence to monocytes as compared to oxygenated sickle cells and oxygenated or deoxygenated normal cells. Deoxygenated sickle RBC–monocyte rosettes were confirmed by microscopy (Fig. 2). This increase in deoxygenated sickle RBC–monocyte adherence correlated with the known pattern of erythrocyte membrane phosphatidylethanolamine and PS bilayer redistribution (from the inner to the outer membrane leaflet) upon the induction of sickling by deoxygenation (Table III). In addition, upon reoxygenation of the deoxygenated sickle RBC–monocyte rosettes, a significant loss of adherent RBC (verified by microscopy) occurred, a result that is consistent with the known reversible sickling-induced exposure of outer leaflet aminophospholipids in these cells (9).

The data presented above strongly suggest that monocytes are capable of recognizing some RBC surface component that is always present in ISC and whose exposure can be reversibly induced by deoxygenating and reoxygenating sickle RSC. Because both ISC and deoxygenated RSC are known to contain elevated levels of membrane outer leaflet aminophos-

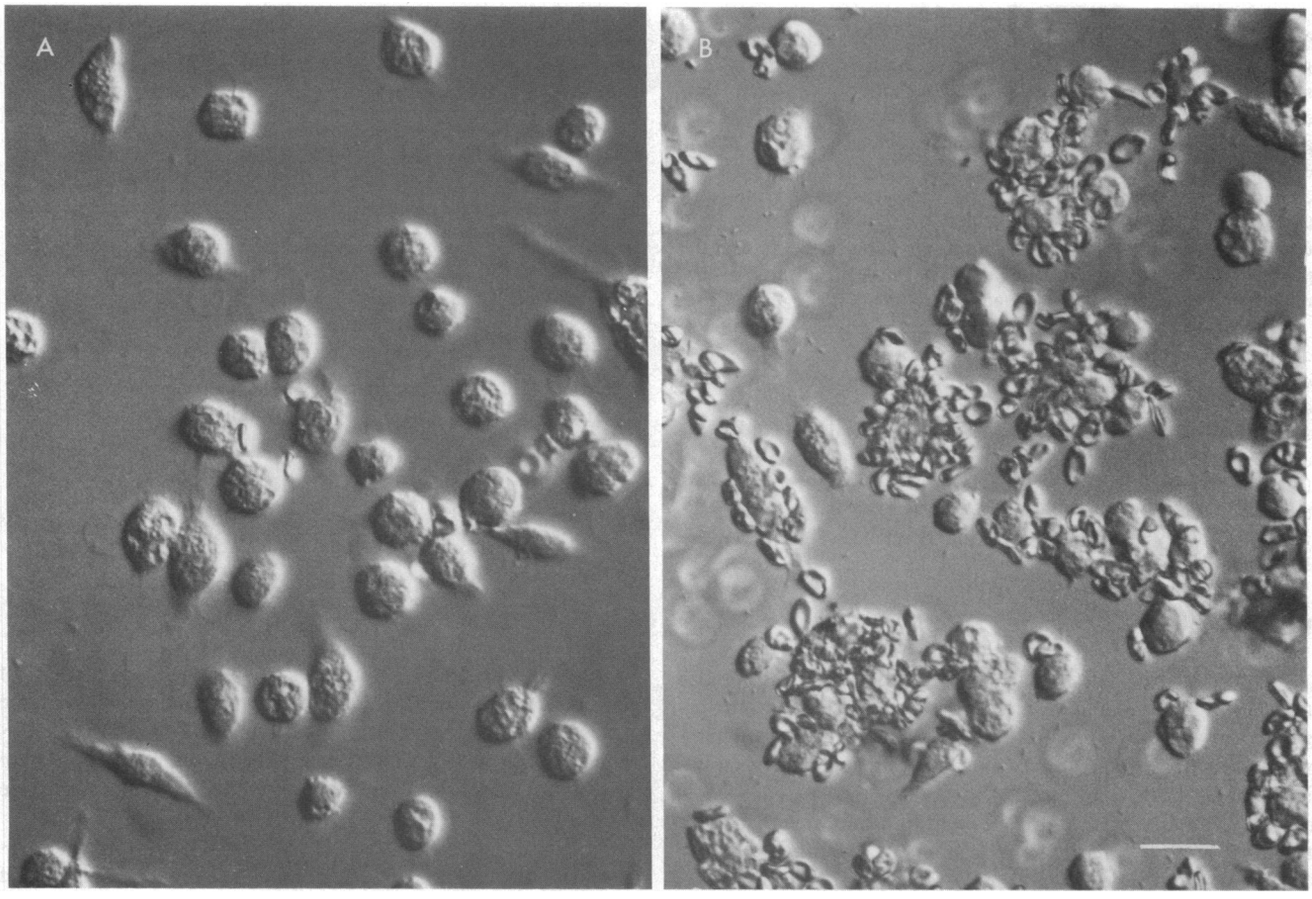

Figure 1. Differential interference-contrast photomicrograph of sickle RBC–monocyte rosettes. Sickle RBC were separated into cohort populations by centrifugation through Stractan-density gradients. Monocyte cultures were incubated with (*A*) ISC-depleted [1% ISC] and (*B*) ISC-enriched [44% ISC] populations for 1 h at 37°C (RBC to monocyte ratio of 100:1), as described in Table I. The coverslips were washed three times with PBS and fixed with 2% paraformaldehyde. Selected field to show adherence characteristics. BAR, 10 μm.

pholipids (9), we speculated that the exposure of aminophospholipids in these cells may result in their increased recognition by and concomitant adherence to human phagocytes.

Incorporation of exogenously supplied PS and PC into RBC. Although the results presented thus far suggest that increased adherence of ISC and deoxygenated RSC to monocytes may be related to the abnormal exposure of membrane outer leaflet aminophospholipids in these cells, they do not rule out the possibility that some other, as yet unidentified, nonphospholipid membrane component becomes exposed in

Table III. Adherence of Deoxygenated RBC to Cultured Peripheral Blood Monocytes

				Erythrocyte membrane outer leaflet lipid concentration			
	% RBC adherence			PE		PS	
RBC	Deoxygenated	Oxygenated	AR: Deoxy/Oxy	Deoxy	Oxy	Deoxy	Oxy
				μM‡	μM‡	μM‡	μM‡
Normal	1.8±1.3	1.3±0.1	1.4 (2.0–0.9)	260	260	0	0
Sickled*	5.7±1.5	0.8±0.5	7.1 (11.7–4.7)	440	260	100	0

Monocytes were isolated from blood and adherence assays were performed as described in Table I, except that the RBC used were ^{51}Cr-labeled oxygenated (Oxy) or deoxygenated (Deoxy) normal or sickle RBC. Subsequent RBC-monocyte incubations and washings were also carried out under oxygenated or deoxygenated (in a sealed chamber constantly flushed with humidified nitrogen) conditions. AR is equal to the ratio of deoxygenated RBC adherence/oxygenated RBC adherence and represents the mean of three individual determinations. Ranges for AR values are given in the parentheses. Simultaneously, the distribution of RBC membrane phospholipids was determined in aliquots of these cells by treatment with bee venom phospholipase A_2, as described previously (9). * 80–90% of the total deoxygenated RBC and 10% of the total oxygenated RBC were morphologically sickled. ‡ Approximate concentration of phospholipid/liter RBC.

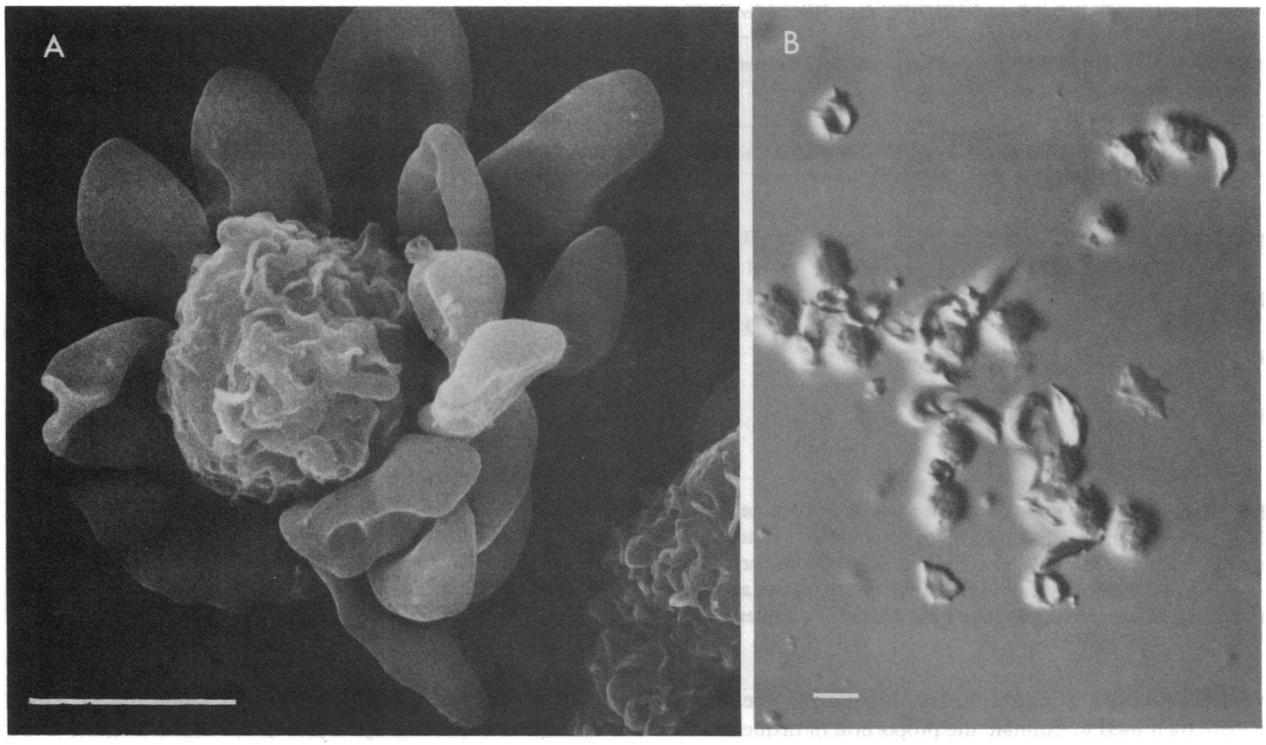

Figure 2. Scanning electron (*A*) and light (*B*) photomicrographs of deoxygenated sickle-RBC monocyte rosettes. Monocyte cultures were incubated with sickle RBC (RBC to monocyte ratio of 100:1) under deoxygenated conditions for 1 h at 37°C, as described in Table I. The coverslips were washed three times with degassed PBS and fixed with 2% paraformaldehyde. Bar, 10 μm.

sickle RBC as a consequence of sickling, which might confer to these cells an increased propensity for adherence to monocytes. Therefore, to define further the physiologic significance that alterations in RBC membrane phospholipid organization may have on RBC recognition by monocytes, we performed similar RBC–monocyte adherence assays using normal RBC whose membrane phospholipid composition was manipulated experimentally. This was accomplished by enriching normal RBC with exogenously supplied phospholipids via incubation with artificial lipid vesicles that contained the transferrable fluorescent phospholipid analogues NBD-PS and NBD-PC.

The addition of NBD-PC or NBD-PS vesicles to a suspension of RBC resulted in rapid fluorescence dequenching (DQ) with maximum fluorescence intensity developing within 1–2 min (Fig. 3). No enhancement of fluorescence occurred in the absence of RBC nor was there an increase in fluorescence after removal of the RBC by centrifugation, which suggests that the observed increase in fluorescence intensity was the result of NBD-phospholipid that had been transferred to and concomitantly diluted into the RBC membrane. These results, however, do not rule out the possibility that in addition to phospholipid transfer, intact vesicles had adhered to the RBC surface. Differentiation between NBD-phospholipid transfer and vesicle adherence was accomplished by comparing fluorescence intensity in the absence of (a measure of NBD-phospholipid transfered to and diluted into the RBC membrane) and presence of (a measure of total RBC-associated NBD-phospholipid) SDS. The measured fluorescence intensities were corrected for differences in the probes' quantum yield (whether the lipid was in RBC, phospholipid vesicles, or detergent micelles), as

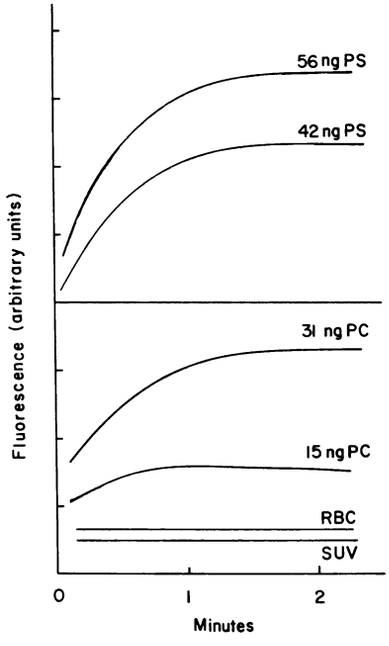

Figure 3. Dequenching of NBD-fluorescence by insertion (dilution) of lipid into RBC membranes. NBD-phospholipid vesicles (15–56 ng of lipid) were rapidly mixed in a cuvette with 1 ml of RBC suspension (10^7 RBC) and the fluorescence (λex 470 nm, λem 525 nm) was continuously monitored at 20°C. Light scattering was prevented by using a high-pass filter (Corning 2A-12, cut-off 510 nm) before the emission monochromator. *Lower traces,* vesicles and RBC alone; *upper traces,* vesicle–RBC mixtures.

Table IV. Insertion of NBD-PS and NBD-PC into RBC Membranes

Vesicle lipid concentration			Relative fluorescence							
			Vesicles		Cells*					
	Initial vesicles	Vesicle-treated RBC	−SDS	+SDS‡	−SDS	+SDS‡	Fv§	Fc§	% DQ‖	Vesicle lipid incorporated
	ng lipid/ml	*ng lipid/ml*								*ng/10^7 RBC*
NBD-PC	125		0.010	0.68			0.015			
	250		0.023	1.53			0.015			
NBD-PS	250		0.028	1.86			0.015			
	425		0.051	3.40			0.015			
NBD-PC		50			0.058	0.060		0.97	97	14
		100			0.107	0.110		0.98	98	23
NBD-PS		40			0.066	0.067		0.99	99	13
		100			0.166	0.168		0.99	99	26

* 10^7 RBC were incubated with the indicated vesicle preparations for 30 min at 37°C. The cells were then washed and fluorescence was measured in the absence and presence of SDS as described in Methods. ‡ Values presented are corrected for sample dilution and appropriate correction factors for SDS-mediated quenching of vesicle and cell fluorescence, respectively. Triplicate samples measured individually. § Fv and Fc are the ratios of fluorescent intensities of the initial vesicles and the washed vesicle-treated RBC in the absence and presence of detergent, respectively (see Methods and reference 7 for details). ‖ Dequenching (DQ) was calculated from Eq. 1.

described previously (7). The corrected fluorescence measurements were then used to estimate the proportion of dequenched RBC-associated NBD-phospholipid in relation to the total amount of RBC-associated NBD-phospholipid (Table IV). By using this procedure we were able to determine that at the concentrations of NBD-PS and NBD-PC used, essentially all (>97%) of RBC-associated fluorescent lipid represented material that had been completely dequenched, which strongly suggests that the lipids were properly inserted into the RBC membrane.

Adherence of PS-enriched RBC to monocytes. ^{51}Cr-labeled normal RBC whose membrane outer leaflet content of PC or PS had been enriched by treatment with NBD-PC or NBD-PS vesicles were incubated with monocytes at 37°C for 1 h. As shown in Table V, RBC enriched with NBD-PS at levels of 26 ng/10^7 RBC (~38 μmol/liter of RBC) were significantly more adherent to monocytes than were RBC enriched with NBD-PC (23 ng/10^7 RBC, ~33 μmol/liter of RBC) or lower levels of NBD-PS (13 ng/10^7 RBC, ~19 μmol/liter of RBC). Control (no addition of phospholipid) RBC had adherence values similar to the NBD-PC-enriched or low-level NBD-PS-enriched RBC. The concentration of PS in the membrane outer leaflet of ISC or deoxygenated RSC is ~100 μmol/liter of RBC; therefore, these results suggest that artificially elevating RBC membrane PS to levels approximately one-third of that found in the outer leaflet of sickled cells can have significant effects on their recognition by and subsequent adherence to monocytes.

Table V. Adherence of PS and PC-enriched RBC to Cultured Peripheral Blood Monocytes

RBC enriched with:	% RBC adherence
NBD-PC, 14 ng/10^7 RBC	1.8±0.6
NBD-PC, 23 ng/10^7 RBC	1.5±0.3
NBD-PS, 13 ng/10^7 RBC	1.7±0.3
NBD-PS, 26 ng/10^7 RBC	3.2±0.5
Control	1.6±0.5

Monocytes were isolated from blood and adherence assays were performed as described in Table I, except that the RBC used were enriched with the phospholipid analogues NBD-PC or NBD-PS, at the levels indicated. Control RBC were treated similarly to enriched cells except that no phospholipid was added. Results are presented as the percentage of treated RBC that remained adherent to the monocytes (mean±SE of five individual determinations).

The data presented above strongly suggest that the mechanism of monocyte recognition of sickled cells may involve a ligand–receptor interaction where RBC surface PS is the ligand recognized by specific PS receptors on the monocytes. To determine whether such PS receptors exist on the monocyte, we preincubated monocyte monolayers with exogenously supplied PS (in the form of small unilamellar liposomes) before the addition of ISC to the monocytes. The adherence of ISC to monocytes pretreated with PS at concentrations >250 μM was reduced by ~60% whereas similar pretreatment with PC at concentrations up to 2.5 mM had no effect on ISC adherence (Table VI). These results suggest that human peripheral blood monocytes possess a receptor that recognizes, and is capable of binding, PS.

Discussion

Accelerated sickle RBC hemolysis is a component in the pathophysiology of sickle cell disease. This increased rate of RBC destruction results in a dramatic decrease in sickle RBC survival, from ~120 days for normal RBC to ~20 days for sickle cells (1). Although the precise nature of the sickle RBC lesion that leads to enhanced RBC hemolysis is unknown, recent observations by Tanaka and Schroit (7) have suggested that the display of outer leaflet PS in PS-enriched mouse RBC might serve as a signal for their recognition by macrophages. This observation prompted us to investigate the possibility of whether a similar mechanism may be operative in sickle RBC,

Table VI. PS-mediated Inhibition of ISC Adherence to Cultured Peripheral Blood Monocytes

Lipid added	ISC adherence
mM	*% of control*
Control	100
PS (0.25)	40±4
PS (0.5)	42±6
PS (2.5)	32±3
PC (0.25)	90±6
PC (0.5)	88±8
PC (2.5)	90±4

Preparation of monocytes and ISC (50%, determined morphologically) and adherence assays were performed as described in Table I, except that monocyte monolayers were preincubated with PS or PC small unilamellar vesicles, at the indicated concentration, for 30 min at 37°C before the addition of ISC. Adherence is expressed as the percentage change in ISC binding to PS or PC pretreated monocytes vs that to control (PBS-treated) monocytes. Results are the mean±SD of seven individual determinations.

where sickling-induced alterations in membrane phospholipid organization results in an increased exposure of outer leaflet PS (9).

Separation of normal and sickle RBC into cohort populations based upon density revealed that the most-dense fractions of sickle RBC (enriched in ISC) were 2.5-fold more adherent to human peripheral blood monocytes than were the most-dense populations of normal RBC, a finding similar to the recent observations of Hebbel and Miller (6) who showed enhanced erythrophagocytosis of dense sickle cells to cultured bone marrow macrophages. We found no significant increase in the adherence of mixed (unfractionated) or least-dense Stractan populations of sickle cells to monocytes, a finding in contrast to that reported by Hebbel and Miller (6), who reported a twofold increase in macrophage erythrophagocytosis of least-dense sickle cell populations. Such differences may be related to different mechanisms involved in the RBC adherence to monocytes vs. RBC erythrophagocytosis by macrophages.

Microscopic examination of monocytes that had been incubated with the dense (ISC-rich) sickle RBC populations revealed the presence of sickle RBC–monocyte rosettes, where the predominant RBC associated with the monocytes appeared morphologically to be ISC. In contrast, examination of monocytes incubated with oxygenated, mixed sickle cell populations, less-dense (ISC-poor) sickle RBC populations, or with any of the density-separated fractions of normal RBC, failed to demonstrate the presence of any significant monocyte–RBC rosette formation, corroborating the previous results of Abramson et al. (12), who found that mixed populations of oxygenated sickle RBC did not form rosettes when added to cultured human leukocyte preparations.

Deoxygenation of sickle RBC induced a dramatic increase in the adherence of these cells to monocytes, whereas similar deoxygenation of normal RBC had essentially no effect on their adherence as compared to the adherence of oxygenated sickle and normal cells, respectively. Exposure of the deoxygenated sickle RBC–monocyte mixture to room air resulted in a significant loss (>90%) of adherent erythrocytes (microscopic observation), which demonstrates the reversibility of sickle RBC–monocyte adherence. Correlating with these reversible changes in sickle RBC adherence was the known reversible alteration in sickle RBC membrane outer leaflet aminophospholipid (PE and PS) content (9). Taken together, these results suggest that the membrane content of RBC outer leaflet aminophospholipids may serve as a trigger for recognition by and adherence to peripheral blood monocytes, a finding strikingly similar to the many observations that cells of the mononuclear phagocyte series avidly recognize synthetic phospholipid vesicles containing PS (13, 14).

These results, however, do not rule out the possibility that other sickle RBC membrane components may also be participating in their enhanced adherence to monocytes. Indeed, sickle RBC are known to contain multiple membrane abnormalities, including elevated levels of surface glycoproteins (15), calcium (16), and hemichrome (17), as well as alterations in the structures of the cytoskeletal proteins (18), increased susceptibility to lipid peroxidation (19), and reduced cellular deformability (20). Petz et al. (21) have recently demonstrated that a subpopulation of sickle RBC contained elevated levels of surface-bound IgG. In that monocytes readily recognize opsonized RBC (12), it is possible that increased adherence of sickle RBC to monocytes is mediated by cell surface immunoglobulin. Indeed, Hebbel and Miller (6) have recently shown partial inhibition of sickle RBC phagocytosis by macrophages following Fc receptor blockade with IgG. However, the exposure and reversibility of the surface component examined in our studies was rapidly inducible by deoxygenation of sickle cells and was reversible by reoxygenation, suggesting that the moiety responsible for increased sickle RBC adherence examined in our studies was probably not antibody related.[2]

To clarify further the role that RBC membrane aminophospholipids might have in promoting sickled RBC–monocyte adherence, we enriched normal RBC with the fluorescent phospholipid analogues NBD-PS and NBD-PC. These NBD-phospholipids are readily transferred from donor vesicles to acceptor cell membranes (7), and the inherent fluorescence self-quenching properties of these compounds allow one to distinguish between genuine phospholipid insertion and uptake of intact phospholipid vesicles. By using this technique, we were able to incorporate NBD-PC or NBD-PS into intact RBC under conditions where binding of intact vesicles to RBC did not occur. Our results indicate that NBD-PS-enriched RBC were more adherent to monocytes than were NBD-PC-enriched or control (PBS-treated) RBC. The amount of incorporated NBD-PS required to enhanced RBC adherence (~38 μmol/liter of RBC) was approximately one-third the level of PS found in the outer leaflet of sickled RBC (~100 μmol/liter RBC, [9]), whereas enrichment of RBC with NBD-PS below this apparent threshold amount had little effect on adherence, strongly suggesting that monocyte recognition of PS-enriched RBC is sensitive to the actual amount of PS in the RBC membrane. Although we have not carried out detailed experiments defining the relationship between the minimum amount of erythrocyte PS required for enhanced adherence to monocytes, one should be aware of the possibility that the actual amount of uniformly dispersed PS in RBC (as obtained in the

2. Whether conditions of low oxygen tension induce an alteration in normal (i.e., oxygenated) monocyte surface characteristics, which might specifically enhance their interaction with sickle cells, is not known.

NBD-PS treated RBC) may not be as crucial as its distribution in the membrane. Indeed, the precise distribution of erythrocyte membrane PS in sickled cells is unknown; one could speculate that localization of exposed PS molecules in sickled cells into specific membrane domains, as has been suggested for erythrocyte membrane PC (22) and phosphatidylethanolamine (23), could lead to an even greater adherence of these cells to monocytes than was measured using the NBD-PS-enriched RBC.

To determine further whether a binding site capable of recognizing PS exists on the monocyte membrane, inhibition studies were performed where monocyte monolayers were preincubated with PS liposomes. Preincubation of the monocytes with PS at concentrations >250 μM resulted in a ~60% inhibition of ISC adherence, whereas similar preincubation with PC at concentrations up to 2.5 mM had no effect on ISC adherence. These findings suggest that a surface moiety on human monocytes may recognize PS, a finding similar to the one reported by Tanaka and Schroit (7) for murine macrophages.

Our findings represent but one example of a broader category of sickle cell properties regarding the enhanced susceptibility of these cells to undergo intermembrane interactions. Other examples of this phenomenon are an increased binding to cultured endothelial cells (3, 4), increased uptake of PS-rich phospholipid vesicles (5), and an enhanced ability of these cells to stimulate in vitro blood coagulation (24).

In conclusion, it appears that abnormal exposure of outer leaflet PS in sickle RBC may significantly affect their propensity for recognition by circulating human phagocytes and may, furthermore, represent a homeostatic mechanism for the removal of pathologic RBC. Although we have not specifically ruled out the involvement of other, as yet unidentified, RBC membrane components in this process, the reversible nature of both adherence to monocytes and expression of surface PS in sickled RBC strongly suggests that alterations in RBC phospholipid asymmetry may have significant pathophysiologic implications.

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