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Research Article

Deoxygenation of erythrocytes from sickle cell anemia (SCA) patients alters membrane phospholipid distribution with increased exposure of phosphatidylethanolamine (PE) and phosphatidylserine (PS) on the outer leaflet. This study investigated whether altered membrane phospholipid exposure on sickle erythrocytes results in complement activation. In vitro deoxygenation of sickle but not normal erythrocytes resulted in complement activation measured by C3 binding. Additional evidence indicated that this activation was the result of the alterations in membrane phospholipids. First, complement was activated by normal erythrocytes after incubation with sodium tetrathionate, which produces similar phospholipid changes. Second, antibody was not required for complement activation by sickle or tetrathionate-treated erythrocytes. Third, the membrane regulatory proteins, decay-accelerating factor (CD55) and the C3b/C4b receptor (CD35), were normal on sickle and tetrathionate-treated erythrocytes. Finally, insertion of PE or PS into normal erythrocytes induced alternative pathway activation. SCA patients in crisis exhibited increased plasma factor Bb levels compared with baseline, and erythrocytes isolated from hospitalized SCA patients had increased levels of bound C3, indicating that alternative pathway activation occurs in vivo. Activation of complement may be a contributing factor in sickle crisis episodes, shortening the life span of erythrocytes and decreasing host defense against infections.

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Activation of the Alternative Complement Pathway by Exposure of Phosphatidylethanolamine and Phosphatidylserine on Erythrocytes from Sick Cell Disease Patients

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Abstract

Deoxygenation of erythrocytes from sickle cell anemia (SCA) patients alters membrane phospholipid distribution with increased exposure of phosphatidylethanolamine (PE) and phosphatidylserine (PS) on the outer leaflet. This study investigated whether altered membrane phospholipid exposure on sickle erythrocytes results in complement activation. In vitro deoxygenation of sickle but not normal erythrocytes resulted in complement activation measured by C3 binding. Additional evidence indicated that this activation was the result of the alterations in membrane phospholipids. First, complement was activated by normal erythrocytes after incubation with sodium tetrathionate, which produces similar phospholipid changes. Second, antibody was not required for complement activation by sickle or tetrathionate-treated erythrocytes. Third, the membrane regulatory proteins, decay-accelerating factor (CD55) and the C3b/C4b receptor (CD35), were normal on sickle and tetrathionate-treated erythrocytes. Finally, insertion of PE or PS into normal erythrocytes induced alternative pathway activation. SCA patients in crisis exhibited increased plasma factor Bb levels compared with baseline, and erythrocytes isolated from hospitalized SCA patients had increased levels of bound C3, indicating that alternative pathway activation occurs in vivo. Activation of complement may be a contributing factor in sickle crisis episodes, shortening the life span of erythrocytes, and decreasing host defense against infections. (*J. Clin. Invest.* 1993. 92:1326–1335.) Key words: phospholipid • membrane • C3 • factor B

Introduction

Sickle cell anemia (SCA)¹ is a genetic disease resulting from a single amino acid change in the β -globin subunit of hemoglobin (1). This abnormal hemoglobin is termed hemoglobin S (Hb S), and homozygous individuals (Hb SS) develop a chronic hemolytic anemia, chronic and progressive tissue and organ damage, and acute painful vasoocclusive crises. Erythrocytes from SCA patients when exposed to low oxygen pressure

become deformed into a sickle shape as a result of polymerization of Hb S within the cell. Sick cell erythrocytes have a shorter life span than normal erythrocytes and are less elastic, more easily trapped in capillaries, and more prone to physiological insults (1, 2). Sick cell erythrocytes also have altered membrane phospholipid distribution after deoxygenation (3–5).

Phospholipids on normal human erythrocytes are asymmetrically distributed across the bilayers of the cell membrane (5, 6). Phosphatidylcholine (PC), sphingomyelin, and < 10% of the total phosphatidylethanolamine (PE) are located in the external leaflet. Most of the PE and all of the phosphatidylserine (PS) are located on the inner leaflet of the membrane bilayer. Deoxygenation of Hb SS erythrocytes results in increased exposure of PE and PS on the outer leaflet (3–5). The abnormal exposure of PS on sickle erythrocytes has been related to increased adherence to endothelial cells (2), procoagulant activity (7), and adherence to mononuclear phagocytic cells (8, 9).

Patients with SCA are susceptible to recurrent bacterial infections, particularly with *Streptococcus pneumoniae* (10). The higher propensity for bacterial infections has been attributed to decreased splenic function (11). Abnormal opsonic activity (12–14) and evidence of alternative complement pathway activation (15–18) have been observed in serum from patients with SCA, suggesting that decreased serum complement may also contribute to the higher risk of bacterial infections. The source of this complement activation has not been determined.

The alternative complement pathway is a primary host defense mechanism, which can be activated by bacterial polysaccharides, virus-infected cells, and other substances in the absence of specific antibodies (19, 20). Upon activation, C3b molecules are deposited covalently on the activator surface leading to opsonization and formation of the lytic membrane attack complex. Liposomes containing PE (21) and/or PS (22–24) activate the alternative complement pathway in human serum. C3b deposition on normal host cells is inhibited by membrane regulatory proteins, including decay-accelerating

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1. Abbreviations used in this paper: CR1, C3b/C4b receptor; DAF, decay-accelerating factor; DPPC, dipalmitoyl-phosphatidylcholine; DPPE, dipalmitoyl-phosphatidylethanolamine; DPPS, dipalmitoyl-phosphatidylserine; GVB, Veronal buffer containing 0.1% gelatin; Hb S, hemoglobin S; Hb SS, hemoglobin S of homozygous individuals; MC540, merocyanine 540; MCP, membrane cofactor protein; NBD-PC, 1-acyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]caproyl]-sn-glycerol-3-phosphocholine; NBD-PE, 1-acyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]caproyl]-sn-glycerol-3-phosphoethanolamine; NBD-PS, 1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]caproyl]-sn-glycerol-3-phosphoserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SCA, sickle cell anemia; VBS, Veronal buffer.

factor (DAF, CD55) (25), membrane cofactor protein (MCP, CD46) (25), and the C3b/C4b receptor (CR1, CD35) (26). DAF, MCP, and CR1 efficiently modulate complement activation by both the classical and alternative pathways by preventing the formation of C3 and C5 convertases on autologous cells and promoting the breakdown of bound C4b and C3b (27–31). Of these, only DAF and CR1 are found on erythrocytes.

The present work has tested the hypothesis that membrane phospholipid changes in deoxygenated sickle erythrocytes lead to complement activation through the alternative pathway. The results indicate that deoxygenated sickle erythrocytes activate the alternative complement pathway in autologous serum and that similar activation can be induced by several other treatments that increase PE and PS exposure on normal erythrocyte membranes. Complement activation occurs despite apparently normal membrane regulatory proteins, DAF and CR1. Analysis of cleaved complement factor B in plasma samples from SCA patients at baseline and in crisis and of bound C3 fragments on erythrocytes from hospitalized and nonhospitalized patients indicate that alternative pathway activation occurs in vivo during sickle crisis episodes.

Methods

SCA patients and normal donors. Venous blood was collected from individuals with Hb SS disease followed at the Duke University Comprehensive Sickle Cell Center's Adult Clinic at the time of routine visit to the clinic or from hospitalized patients for routine blood cell analysis. Homozygous hemoglobin A (Hb AA) donors were normal volunteers.

Erythrocytes. Sheep erythrocytes were purchased from the Colorado Serum Co. (Denver, CO). Human and rabbit venous blood was drawn into EDTA-anticoagulated tubes. Human blood was diluted 1:7 with acid-citrate-dextrose solution and kept at 4°C for < 48 h before use in complement activation assays. Erythrocytes were washed and resuspended in veronal buffer solution (VBS; containing [mM] 150 sodium chloride, 3 sodium bicarbonate, 2.5 barbituric acid, and 1.5 sodium diethylbarbiturate, pH 7.4) before each experiment. Human erythrocyte preparations contained < 0.1% leukocytes as determined by flow cytometry using Simultest LeucoGATE® (Becton-Dickinson & Co., Mountain View, CA).

Serum and plasma. Human plasma was prepared by centrifugation of citrated blood. Serum was isolated by centrifugation of blood collected into glass tubes and allowed to coagulate in vitro. Plasma and serum were stored in aliquots at –70°C immediately after separation.

Complement components. Human factors B (32), H (33), P (34), and C3 (35), C1 (36), and factor D (37) were purified as described. Functionally purified factor I (35) was further purified on an affinity matrix prepared using goat antifactor I (Quidel, San Diego, CA) and CarboLink® coupling gel (Pierce Chemical Co., Rockford, IL). Human C2 and C4 were purchased from Quidel. Purified C3 was labeled with [¹²⁵I]sodium iodide (ICN Biochemicals, Inc., Costa Mesa, CA) to a specific activity of ~ 0.1 µCi/µg using Iodo-beads® (Pierce Chemical Co.).

Complement activation by human erythrocytes. Autologous serum containing 2.5 mM MgCl₂ and 10 mM EGTA to block the classical pathway and permit activation of the alternative pathway was added to an equal volume of erythrocyte suspension at a concentration of 10⁹ cells/ml. Control samples were incubated with autologous serum chelated with 10 mM EDTA to block both complement pathways. Serum and erythrocyte mixtures were incubated for 30 min at 37°C, washed with VBS containing 0.1% gelatin (GVB), and lysed in PBS containing either 0.5% Triton X-100 or 0.5% NP-40. Samples were stored at –70°C before testing C3 deposition.

The purified alternative complement pathway was prepared using C3, MgCl₂, and factors B, D, H, I, and P at their physiological concen-

trations in GVB as previously described (38). Human erythrocytes at 10⁹ cells/ml in VBS were incubated with an equal volume of the purified component mixture for 30 min at 37°C. After incubation, erythrocytes were washed, lysed, and frozen before testing C3 deposition.

C3 binding assays. A competitive ELISA using horseradish peroxidase-conjugated anti-C3 antibody (Cappel Laboratories, Durham, NC) was used to quantitate C3 deposition on erythrocytes as previously described (21). The amount of C3 bound to erythrocytes was calculated from a standard curve prepared using 0.03–8 µg/ml purified C3. Values from EDTA controls were subtracted. The assay detects C3d as well as intact C3, C3b, and iC3b. The molecular weight of C3b was used to calculate molecules bound per cell. For experiments to assess the presence of C3 fragments on washed erythrocytes without serum incubation, washed erythrocytes were lysed at a concentration of 6.7 × 10⁸ cells/ml and added to the ELISA. Rabbit erythrocytes prepared in the same way were used to determine a negative control value, which was subtracted from the data.

The same washed erythrocytes were tested for the presence of C3 by a flow cytometric assay. 10⁸ erythrocytes were incubated with polyclonal goat anti-human C3 (Quidel) that had been affinity purified by absorption and elution from C3b bound to activated thiol Sepharose®, washed with GVB, and incubated with FITC-conjugated rabbit anti-goat IgG (Sigma Chemical Co., St. Louis, MO). Controls were incubated with second antibody only or with no antibody. Fluorescence was measured on an EPICS C® fluorescence-activated cell sorter (Coulter Corp., Hialeah, FL). Staining observed with the FITC-conjugated secondary antibody was subtracted from staining observed with both antibodies for each sample using Immunoanalysis software (Coulter Corp.). Staining with the second antibody was not significantly greater than autofluorescence.

In vitro deoxygenation/oxygenation of erythrocytes. Human erythrocytes resuspended in VBS were gently bubbled with pure nitrogen (deoxygenated) or oxygen (oxygenated) at room temperature for 5 min. Erythrocyte suspensions were then incubated either sealed (deoxygenated) or not sealed (oxygenated) for 2 h at 37°C. After incubation, cells were washed again and resuspended in VBS to a final concentration of 10⁹ cells/ml before incubation in autologous serum.

Tetrathionate treatment of erythrocytes. Human erythrocytes from Hb AA donors were incubated with sodium tetrathionate, a thiol-oxidizing agent, as described (39). 1 ml of packed erythrocytes was resuspended in 9 ml of a buffer solution containing the following (mM): 90 KCl, 45 NaCl, 10 sodium phosphate, and 44 sucrose (pH 8.0). Cell suspensions were incubated at 37°C with different concentrations of sodium tetrathionate for the times indicated in the results. After incubation, cells were washed and resuspended in the same buffer. A fluorescent probe, merocyanine 540 (MC540; Sigma Chemical Co.), was used to measure membrane phospholipid changes on sodium tetrathionate-treated erythrocytes as previously described (40). Increased binding of MC540 correlates with increased exposure of inner leaflet phospholipids containing unsaturated fatty acyl side chains on the outer leaflet of erythrocyte membranes (40) and has been shown to correlate with measurement of PE and PS exposure on erythrocytes by other methods. Cell suspensions were incubated with MC540 at a final concentration of 4 µg/ml for 4 min at room temperature. After incubation, the binding of MC540 was measured directly using a spectrofluorophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD) (excitation λ, 570 nm; emission λ, 612 nm).

Assay for total serum alternative complement pathway. Serum was assayed for alternative pathway activity by a hemolytic assay (41). Serum samples serially diluted in VBS containing 2.5 mM MgCl₂ and 10 mM EGTA were incubated with rabbit erythrocytes for 30 min at 37°C. VBS containing 10 mM EDTA was added to stop the activation, samples were centrifuged, and absorbance of supernatants was read at 541 nm for hemolysis. The serum dilutions resulting in 50% hemolysis (CH₅₀) were calculated as previously described (41).

DAF and CR1 antigen levels. DAF and CR1 expression on human erythrocytes were determined using anti-DAF mAb IIH6 (42), or anti-CR1 (Becton-Dickinson & Co.) and FITC-conjugated F(ab')₂

goat anti-mouse IgG (Caltag, South San Francisco, CA) on a FAC-Scan® flow cytometer.

Functional activity of DAF. DAF was purified from human erythrocytes as described (43). Purified DAF was incorporated into complement-activating liposomes containing dipalmitoyl-phosphatidylethanolamine (DPPE), dipalmitoyl-phosphatidylcholine (DPPC), and cholesterol (molar ratio 4:6:5) at a concentration of 1 μ g of DAF/ μ mol phospholipid as previously described (44). Liposomes were incubated at 37°C for 1 h in the presence of 20 mM sodium tetrathionate. After treatment, liposomes were incubated with normal human serum, washed, lysed, and C3 deposition was determined by ELISA. Tetrathionate-treated liposomes were compared with untreated liposomes containing or lacking DAF.

The functional activity of DAF in human erythrocytes was measured by its ability to inhibit the classical pathway C3 convertase, C4b2a (43). DAF inhibits this convertase by dissociating C2a from C4b (45). Human erythrocytes (100 μ l at 10^9 cells/ml) were incubated sequentially with rabbit anti-human erythrocyte serum (final concentration 1:1600) (Cappel Laboratories), human euglobulin C1 (20 μ g), human C4 (4 μ g), and human euglobulin C1 (20 μ g), with washing between each step, to form huEAC14b. To assess C3 convertase function, 7.5 ng oxidized human C2 (^{oxy}C2) (43, 46) was added to 2.5×10^7 huEAC14b in 50 μ l GVB²⁺ (GVB containing 0.15 mM CaCl₂ and 0.5 mM MgCl₂) and incubated for 5 min at 30°C to form the C3 convertase. Then 100 μ l of [¹²⁵I]C3 (50 μ g) was added directly to the mixture in 100 μ l GVB containing 10 mM EDTA (to prevent further convertase formation), and the cells were incubated for 30 min at 37°C. After incubation, cells were washed three times and bound [¹²⁵I]C3 was determined by counting the pellets using a gamma counter (Minaxi®; Packard Instruments Co. Inc., Downers Grove, IL). As controls, normal erythrocytes were incubated for 30 min on ice with anti-DAF mAbs (IA10, IIH6, and VIIIA7) (42, 43) to inhibit DAF function before incubation with rabbit anti-human erythrocyte antibody, C1, C4, and C2, and measurement of [¹²⁵I]C3 binding. Anti-DAF treatment did not affect the amount of C4 bound to the erythrocytes after antibody, C1, and C4 incubations.

Functional activity of CRI. The function of CRI was determined by its cofactor activity for factor I-dependent cleavage of bound C3b (47, 48). 10^7 sheep erythrocytes with bound [¹²⁵I]C3b deposited by the classical pathway as described (48) were incubated with 2×10^7 human erythrocytes in the presence of 1 μ g of purified factor I for 30 min at 37°C. After incubation, supernatants were analyzed by 5–15% gradient SDS-PAGE and autoradiography. CRI function on erythrocytes from different individuals was compared by the density of the released 27-kD C3c fragment on the autoradiograph.

Insertion of phospholipids into erythrocytes. Synthetic dipalmitoyl-phosphatidylserine (DPPS) was purchased from Sigma Chemical Co. Synthetic DPPC and DPPE were purchased from Calbiochem Corp. (La Jolla, CA). 1-Palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]caproyl]-sn-glycerol-3-phosphoserine (NBD-PS), 1-acyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]caproyl]-sn-glycerol-3-phosphocholine (NBD-PC), and 1-acyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]caproyl]-sn-glycerol-3-phosphoethanol-amine (NBD-PE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL).

Phospholipid mixtures made of DPPC/NBD-PC, DPPS/NBD-PS, or DPPE/NBD-PE with molar ratio 4:1, 4:1, and 29:1 respectively, were dissolved in pure chloroform and dried under nitrogen. Vesicles were then prepared by sonication of 5 μ g/ml (DPPC/NBD-PC and DPPS/NBD-PS) or 30 μ g/ml (DPPE/NBD-PE) in PBS for 10 min at room temperature with a probe sonicator (Ultrasonic Power; Freeport, IL) as described (8). Contaminating large multilamellar vesicles were removed from the suspension by centrifugation at 2,000 *g* for 15 min.

Phospholipid vesicles were incubated with erythrocyte suspensions for 30 min at 37°C (8). After incubation, erythrocytes were washed and resuspended at 10^9 cells/ml in VBS before autologous serum incubation. The mean channel fluorescence of treated erythrocytes was measured by flow cytometry using fluorescein settings. Phospholipid

incorporation was then calculated from standard curves of mean channel fluorescence versus incorporation for each phospholipid. To construct the standard curves, phospholipid incorporation for a range of vesicle/erythrocyte ratios was determined by fluorimetry of fully dequenched unincorporated vesicles compared with pure phospholipid standards (excitation λ , 470 nm; emission λ , 525 nm) and plotted against the mean channel fluorescence readings of the erythrocytes. Insertion of labeled lipid into erythrocyte membranes was confirmed by visual observation of bright uniform rings of membrane fluorescence in treated cells. To determine whether adherent vesicles were present in the treated erythrocyte preparations, fluorescence (excitation λ , 470 nm; emission λ , 525 nm) of treated erythrocytes was determined in the presence and absence of 2% Triton X-100. Because of the self-quenching properties of the labeled phospholipids, the phospholipid vesicles show about a 10-fold increase in fluorescence after lysis (8). Lipid that has inserted into the erythrocyte membrane by contrast is dequenched by dilution with membrane lipids and its fluorescence is not increased by detergent solubilization. The erythrocytes used for complement activation studies did not show increased fluorescence after lysis, indicating minimal contamination with intact vesicles.

Plasma Bb. Plasma Bb concentration was measured using a commercial ELISA from Quidel. Paired plasma samples were obtained from three SCA patients at baseline and during acute sickle crisis episodes. Patients were considered to be at baseline if they had no pain or fever at the time of presentation and returned a postcard 48 h later stating that they had not experienced pain or fever in the 48 h after their samples were drawn. Patients were considered to have pain crisis when they presented to the emergency department or clinic with pain that was consistent with their usual sickle cell pain crisis; no other cause was found for the pain; and there was no resolution of pain after 8 h of treatment with parenteral narcotics. Five additional plasma samples from three other SCA patients with chronic pain were also analyzed.

Statistical analysis. Comparisons between means were made using the *t* test with *P* < 0.05 considered to be significantly different.

Results

Activation of the alternative complement pathway by deoxygenated erythrocytes from SCA patients. Deoxygenated or oxygenated erythrocytes obtained from SCA patients during routine clinic visits and healthy controls were incubated with MgEGTA-treated autologous serum and alternative pathway activation determined by C3 deposition (Fig. 1). C3 was

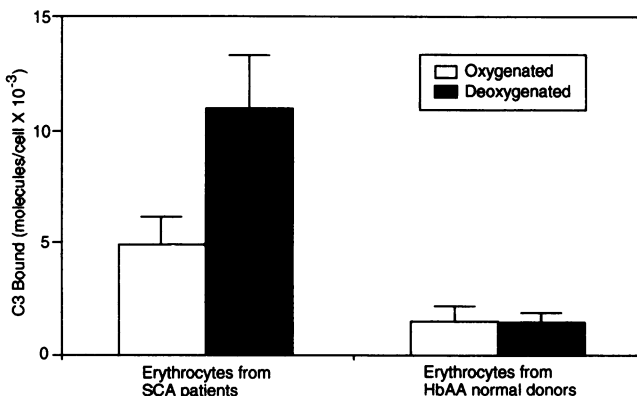


Figure 1. Alternative pathway activation in autologous serum by deoxygenated sickle erythrocytes. Erythrocytes from normal donors or SCA patients were incubated with MgEGTA-treated autologous serum after in vitro deoxygenation or oxygenation. Cells were washed, lysed, and deposited C3 was measured by a competitive ELISA. The means \pm SEM are shown for nine SCA patients and four normal donors.

bound to erythrocytes from homozygous Hb SS patients ($n = 9$), and there was a more than twofold increase in C3 bound on deoxygenated compared with oxygenated erythrocytes. No hemolysis was observed after serum incubation. Hb AA erythrocytes ($n = 4$) showed very little complement activation after the same deoxygenation or oxygenation treatments. The C3 deposition required *in vitro* complement activation since only a minimal amount of C3 was detected on sickle or normal erythrocytes after incubation with EDTA-treated serum (data not shown). Values for erythrocytes incubated with EDTA-treated serum were $< 1,000$ molecules/cell and have been subtracted from the data shown. Oxygenated sickle erythrocytes showed greater C3 binding than Hb AA erythrocytes, which may be due to senescent or irreversibly sickled erythrocytes within the total erythrocyte population. The C3 bound to sickle erythrocytes was found to be predominantly C3b and/or iC3b by substituting mouse mAb 129, which recognizes specifically human C3b and iC3b (38) for the polyclonal anti-C3 antibody in the ELISA.

Total alternative pathway levels in serum from SCA patients. Since previous reports have shown decreased alternative pathway activity in serum from SCA patients (16, 49, 50), the functional activity of the alternative pathway was determined by hemolysis of rabbit erythrocytes. The total alternative pathway activity in CH_{50} units was 3.93 ± 0.22 U/ml for serum from SCA patients ($n = 9$) and 3.77 ± 0.22 U/ml for serum from normal donors ($n = 8$). These values for healthy SCA patients and normal donors did not differ significantly ($P > 0.05$).

Membrane phospholipid changes and activation of the alternative complement pathway after tetrathionate treatment of Hb AA erythrocytes. Incubation of normal erythrocytes with thiol-oxidizing agents, including sodium tetrathionate and diamide, results in increased PE and PS exposure on the outer membrane leaflet (39, 51) similar to the phospholipid changes observed in deoxygenated erythrocytes from Hb SS patients. To determine whether altered membrane phospholipid distri-

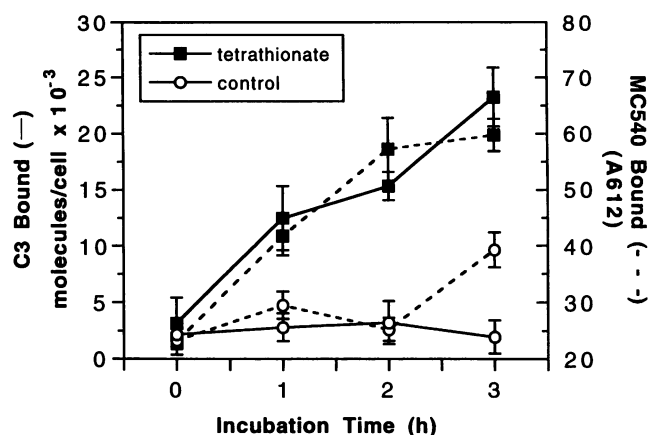


Figure 2. Membrane phospholipid redistribution and complement activation on normal erythrocytes after treatment with sodium tetrathionate. Erythrocytes were treated with 5 mM sodium tetrathionate or buffer for 0, 1, 2, or 3 h at 37°C. MC540 binding that correlates with PE and PS exposure was measured fluorimetrically (dotted lines). Treated erythrocytes were incubated with MgEGTA-treated autologous serum, washed, lysed, and deposited C3 was measured by a competitive ELISA (solid lines). Circles are buffer-treated erythrocytes. Squares are tetrathionate-treated erythrocytes. Means \pm SEM are shown for four experiments.

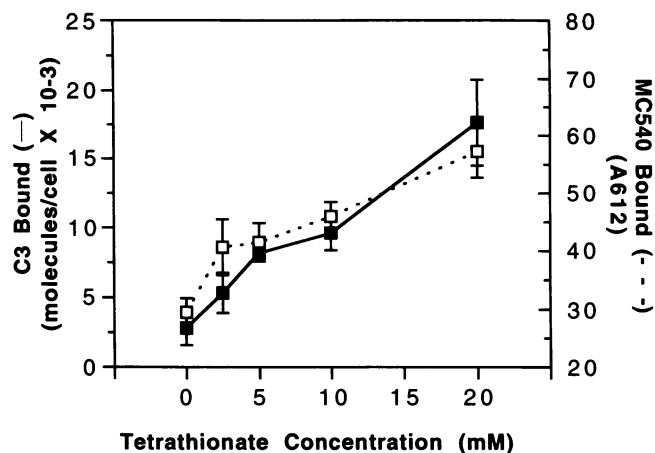


Figure 3. Membrane phospholipid redistribution and complement activation on normal erythrocytes after treatment with sodium tetrathionate. Erythrocytes were treated with 0–20 mM sodium tetrathionate for 1 h at 37°C. MC540 binding that correlates with PE and PS exposure was measured fluorimetrically (open squares, dotted lines). Treated erythrocytes were incubated with MgEGTA-treated autologous serum, washed, lysed, and deposited C3 was measured by a competitive ELISA (closed squares, solid lines). Means \pm SEM are shown for four experiments.

bution in normal erythrocytes would induce complement activation, Hb AA erythrocytes were treated with 5 mM sodium tetrathionate for 0–3 h or with 2.5–20 mM sodium tetrathionate for 1 h at 37°C. Membrane phospholipid changes were monitored by fluorimetry using MC540, which binds to inner leaflet phospholipids. Increased binding of MC540 has been shown to correlate with exposure of PE and PS (40). MC540 binding increased with incubation time (Fig. 2) and sodium tetrathionate concentration (Fig. 3). A corresponding increase in complement activation by tetrathionate-treated erythrocytes after incubation in MgEGTA-treated autologous serum was observed (Figs. 2 and 3). Erythrocytes from the same donors incubated in buffer showed little increase in MC540 binding or C3 deposition.

Activation of the purified alternative pathway by tetrathionate-treated and sickle erythrocytes. Naturally occurring antibody that binds to clustered band 3 on senescent and diamide-treated erythrocytes has been reported to enhance complement activation (52). To determine whether antibody was required for complement activation by tetrathionate-treated erythrocytes, erythrocytes from Hb AA normal donors were treated with 5 mM tetrathionate for 3 h or 20 mM tetrathionate for 1 h and C3 deposition measured after incubation with the six purified components of the alternative pathway. As shown in Fig. 4 a, tetrathionate-treated erythrocytes activated complement in this purified mixture in the absence of antibody. Deoxygenated sickle erythrocytes also activated complement after incubation with purified alternative pathway components (Fig. 4 b). Tetrathionate-treated erythrocytes activated complement in serum that had been absorbed with tetrathionate-treated erythrocytes to remove antibody to band 3 or other erythrocyte molecules (data not shown).

Antigenic and functional levels of DAF and CR1 in sickle and tetrathionate-treated erythrocytes. To determine whether decreased levels or activities of regulatory proteins contributed to the complement activation observed after deoxygenation or

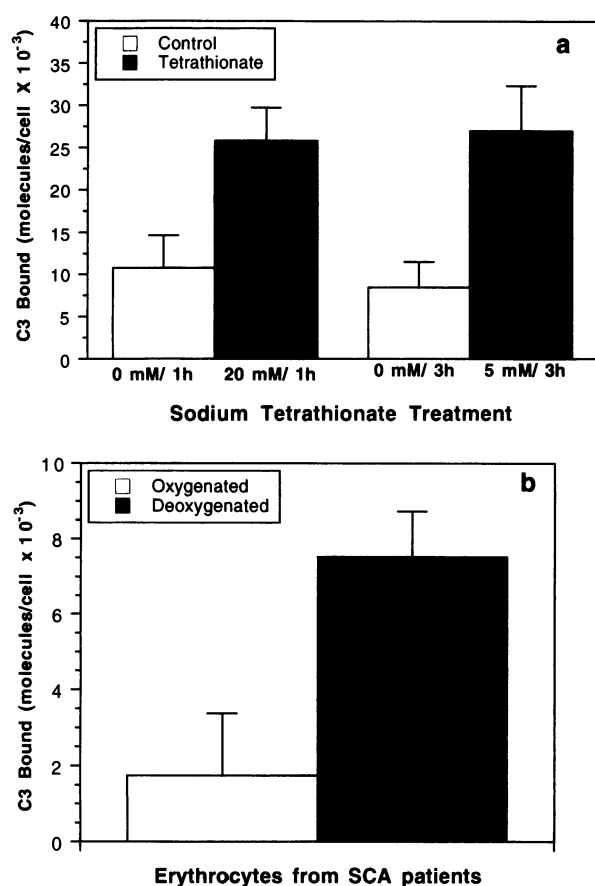


Figure 4. Activation of the purified alternative pathway by (a) erythrocytes treated with sodium tetrathionate or (b) erythrocytes from SCA patients, deoxygenated or oxygenated in vitro. Erythrocytes were incubated with purified alternative pathway components after in vitro treatments. Cells were washed and lysed, and deposited C3 was measured by a competitive ELISA. In a, open bars are control cells incubated in the same buffer without sodium tetrathionate. Means \pm SEM are shown for three experiments.

tetrathionate treatment, the antigenic and functional levels of DAF and CR1 were studied. As shown in Table I, antigenic levels of DAF and CR1 on erythrocytes were not different in erythrocytes from SCA and normal donors and were not changed by in vitro deoxygenation or tetrathionate treatment.

To test for a direct effect of tetrathionate treatment on DAF

function, liposomes composed of PE, PC, and cholesterol were prepared with and without incorporated DAF. Liposomes without DAF activated the alternative complement pathway and showed C3 binding ($3.7 \mu\text{g}/\mu\text{mol}$ phospholipid) after serum incubation (Fig. 5). DAF inhibited C3 binding by liposomes of the same composition by 84%. DAF inhibited C3 binding by 96% after treatment of liposomes with 20 mM tetrathionate for 1 h, demonstrating that purified DAF is not inactivated by sodium tetrathionate treatment.

The functional activity of membrane DAF was also measured in deoxygenated sickle and tetrathionate-treated erythrocytes by its ability to inhibit the classical pathway C3 convertase (Table II). DAF dissociates the classical pathway C3 convertase, decreasing C3 binding to the cells as shown by the dramatic increase in [^{125}I]C3 binding to human erythrocytes treated with anti-DAF before formation of the convertase. To control for possible differences in convertase formation, bound C4 was assayed by ELISA on lysed samples of cells before incubation with C3. There were no differences in bound C4 for any groups including anti-DAF-treated cells (342 C4 molecules/cell) compared with untreated controls (400 molecules/cell). Deoxygenated sickle erythrocytes did not show significantly greater C3 binding than oxygenated sickle erythrocytes or erythrocytes from normal donors. Treatment of normal erythrocytes with 20 mM tetrathionate for 1 h (Table II) or 5 mM tetrathionate for 3 h (data not shown) also did not increase C3 binding compared with control cells. Thus, complement activation did not correlate with decreased DAF function.

The cofactor function of erythrocyte membrane CR1 was tested by incubating sheep erythrocytes bearing [^{125}I]C3b with human erythrocytes and purified factor I and measuring the release of the cleavage product C3c. CR1 cofactor activity on deoxygenated sickle erythrocytes from six patients and tetrathionate-treated normal erythrocytes was not different from the activity of erythrocytes from normal donors (Fig. 6 and Table III). C3c release required both factor I and human erythrocytes (data not shown).

Alternative pathway activation after insertion of PE and PS into normal erythrocytes. As a method of altering membrane phospholipid composition without disrupting the cytoskeleton, normal erythrocytes were incubated with synthetic phospholipid vesicles to increase PE, PS, or PC concentration in the membrane. Insertion was measured with fluorescent phospholipids. As shown in Fig. 7, Hb AA erythrocytes activated the alternative pathway in autologous serum after insertion of low concentrations of PE or higher concentrations of PS, but not

Table I. Antigenic Levels of DAF and CR1 on Erythrocytes Determined by Flow Cytometry Using Monoclonal Anti-DAF and Anti-CR1 Antibodies

Donors	Treatment	DAF*	n	CR1*	n
Normal donors	None	288 \pm 8	4	108 \pm 35	3
SCA patients	Deoxygenation	290 \pm 10	10	90 \pm 3	9
SCA patients	Oxygenation	296 \pm 9	10	106 \pm 6	9
Normal donors	20 mM Tetrathionate for 1 h	319 \pm 21	4	77 \pm 16	3
Normal donors	Buffer control for 1 h	314 \pm 18	4	77 \pm 21	3
Normal donors	5 mM Tetrathionate for 3 h	320 \pm 22	4	72 \pm 14	3
Normal donors	Buffer control for 3 h	303 \pm 19	4	68 \pm 20	3

* Mean channel fluorescence \pm SEM.

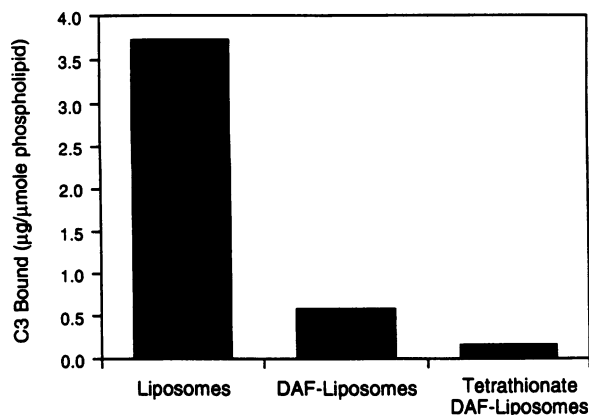


Figure 5. Functional activity of purified DAF after tetrathionate treatment. Purified DAF was incorporated into liposomes containing DPPE, DPPC, and cholesterol (molar ratio 4:6:5) at a concentration of 1 µg of DAF/µmol phospholipid. DAF-containing liposomes were incubated at 37°C for 60 min in the presence of 20 mM sodium tetrathionate. After treatment, liposomes were incubated with normal human serum, washed, lysed, and C3 deposition was determined by ELISA. Tetrathionate-treated liposomes containing DAF were compared with untreated liposomes containing or lacking DAF. Means of duplicate samples are shown.

PC. In these experiments, the maximum amount of PE insertion was 2 µg/10⁹ cells whereas the insertion for PC and PS reached a final concentration of > 13 µg/10⁹ cells. These maximum incorporation levels represent increases of ~ 2% of total PE and 18% of total PS, calculated as described (4, 8). No hemolysis was observed after phospholipid insertion at these concentrations. Adherent intact liposomes were not detected in the erythrocyte preparations by measurement of fluorescence dequenching after lysis (see Methods).

Evidence for *in vivo* alternative complement pathway activation in SCA patients. To determine whether complement activation occurs *in vivo* in SCA, plasma samples from SCA patients and normal donors were tested for the presence of cleaved factor B (Bb). Bb is generated from factor B by factor D during formation of the alternative pathway C3 convertase. Previous analyses in our laboratory have found a mean plasma Bb concentration of 0.70±0.18 µg/ml for 12 normal donors. In the present study plasma samples obtained from three normal Hb AA donors assayed on the same plate as the SCA samples showed a Bb concentration of 1.44±0.34 µg/ml (Fig. 8). Paired plasma samples isolated from three SCA patients demonstrated a twofold higher Bb fragment concentration in crisis compared with baseline ($P = 0.037$). Bb levels in samples from SCA patients at baseline were not significantly higher than normal controls ($P > 0.05$). Five SCA patients characterized clinically as suffering from chronic pain also showed increased plasma Bb concentrations compared with normal controls ($P = 0.001$).

Erythrocytes with bound fragments of C3 (C3d and C3dg) have been reported in several hemolytic anemias and systemic autoimmune diseases (53). Although initial experiments indicated that the amount of C3 bound to cells from healthy SCA patients was very low, we examined erythrocytes from a larger number of hospitalized and nonhospitalized SCA patients for bound C3 using the competitive C3 ELISA (Fig. 9) and flow cytometry with anti-C3 antibody. The results of both assays

showed significantly increased C3 on erythrocytes from patients compared with controls. By ELISA, control erythrocytes had an average of 74±23 C3 molecules per cell, erythrocytes from outpatients had 369±122 C3 molecules per cell, and erythrocytes from hospitalized patients had 855±147 C3 molecules per cell. These values were significantly higher for hospitalized patients compared with controls ($P = 0.0001$), hospitalized patients compared with outpatients ($P = 0.028$), and outpatients compared with controls ($P = 0.021$). By flow cytometry erythrocytes from hospitalized patients and outpatients had a significantly higher percentage of cells stained by anti-C3 antibody and significantly higher mean fluorescence of the positive cells compared with erythrocytes from normal donors (data not shown). These results and the Bb data (Fig. 8) indicate that alternative pathway activation in SCA patients correlates with periods of crisis and that SCA erythrocytes are involved in this activation.

Discussion

This study demonstrates activation of the alternative complement pathway in autologous serum by deoxygenated sickle erythrocytes. Several lines of evidence indicate that this complement activation is the result of the increased appearance of PE and PS in the outer membrane leaflet of these cells.

Alternative pathway activation is initiated by the binding of C3b to surfaces (19, 20). The bound C3b then assembles the alternative pathway C3 convertase, C3bBb, by the binding of factor B and its cleavage by factor D. The C3 convertase cleaves additional C3 creating a positive amplification loop for the deposition of C3b on the activating surface. Membrane-bound C3b molecules facilitate phagocytosis and can further activate C5 through C9 to form lytic membrane attack complexes. The alternative pathway is a tightly regulated system in which inter-

Table II. The Functional Activity of Erythrocyte Membrane DAF Assayed by Inhibition of C3 Convertase Activity

Donors	Treatment	C3 bound*	n
Normal donors	Anti-DAF mAbs [‡]	199,455±42,857	9
Normal donors	None	3,566±846	10
SCA patients	Deoxygenation	3,667±1,260	4
SCA patients	Oxygenation	4,114±1,020	4
Normal donors	20 mM Tetrathionate for 1 h	5,874±2,288	6
Normal donors	Buffer control for 1 h	3,156±100	6

* cpm±SEM. Human erythrocytes were treated with rabbit antibody, human C1, C4, and ¹²⁵I-C2 to form a C3 convertase. DAF function was determined by inhibition of [¹²⁵I]C3 binding. There was no significant difference in C3 binding between sickle and normal erythrocytes, between deoxygenated and oxygenated sickle erythrocytes, or between normal or buffer control and sodium tetrathionate-treated erythrocytes (all $P > 0.05$). [‡] Erythrocytes were pretreated with three anti-DAF mAbs to inhibit DAF function.

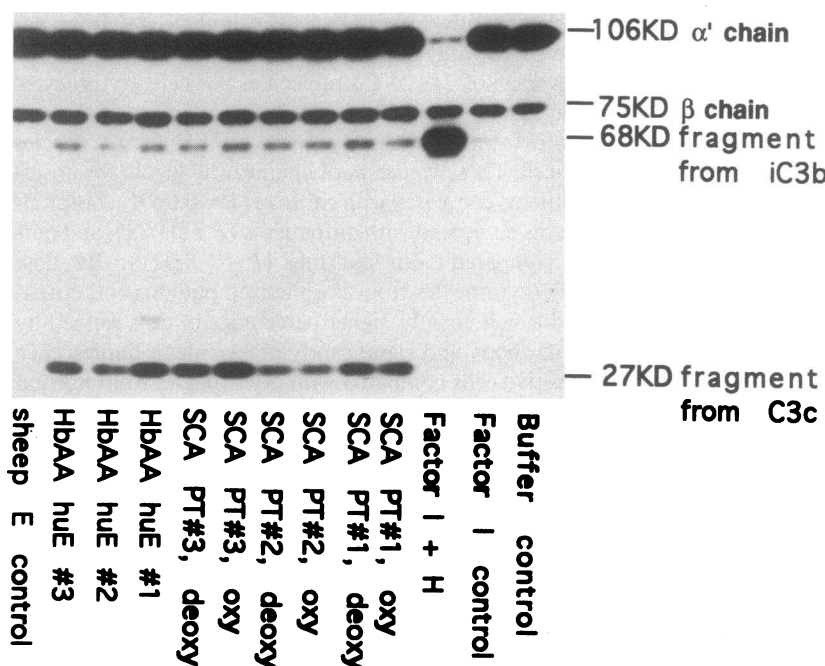


Figure 6. Functional activity of CR1 on Hb AA and Hb SS erythrocytes. 10^7 sheep erythrocytes with bound ^{125}I C3b were incubated for 30 min at 37°C with $1\text{ }\mu\text{g}$ purified factor I and 2×10^7 normal erythrocytes or sickle erythrocytes after in vitro deoxygenation or oxygenation. Cells were centrifuged and supernatants were analyzed by 5–15% gradient SDS-PAGE and autoradiography for cleavage of C3b. Control lanes were ^{125}I C3b sheep erythrocytes incubated with buffer alone, factor I alone, factor I and $5\text{ }\mu\text{g}$ H (to produce iC3b), and factor I and sheep E (lacking CR1). Results of this and additional experiments are summarized in Table III.

actions between the activating surface, C3b, and regulatory proteins determine whether amplification will take place.

Previous results from our laboratory (21) and others (22, 23, 54) have shown that liposomes containing PE or PE and PS or inside-out vesicles prepared from erythrocytes activate the alternative complement pathway. Thus, it seemed possible that

Table III. The Functional Activity of Erythrocyte Membrane CR1 Assayed by Cofactor Activity of the Cleavage of C3b by Factor I

Donors	Treatment	C3c released*	n
Normal donors	None	3.185 ± 0.281	4
SCA patients	Deoxygenation	3.027 ± 0.197	6
SCA patients	Oxygenation	3.125 ± 0.254	6
Normal donors	20 mM Sodium tetrathionate for 1 h	4.372 ± 0.287	6
Normal donors	Buffer control for 1 h	4.747 ± 0.164	6
Normal donors	5 mM Tetrathionate for 3 h	4.084 ± 0.334	4
Normal donors	Buffer control for 3 h	4.342 ± 0.278	6

* Density of 27-kD band \pm SEM. Sheep erythrocytes were treated with rabbit antibody, human C1, C4, $^{55}\text{C}2$, and ^{125}I C3. Erythrocytes were assayed for CR1 cofactor activity by adding human erythrocytes to the sheep cell intermediates in the presence of factor I. Released C3c was analyzed by the appearance of a 27-kD band from C3c in supernatant samples by SDS-PAGE and autoradiography. There was no significant difference in CR1 activity between sickle and normal erythrocytes, between deoxygenated and oxygenated sickle erythrocytes, or between control and sodium tetrathionate-treated erythrocytes (all $P > 0.05$).

the membrane phospholipid changes associated with deoxygenation of sickle erythrocytes (3–5) were responsible for the complement activation we observed when these cells were incubated with autologous serum. In support of this hypothesis, two other treatments that increase PE and PS exposure were shown to cause complement activation by normal erythrocytes. Thiol-oxidizing agents, including sodium tetrathionate and diamide, induce membrane phospholipid redistribution by disruption of the cytoskeleton (55) whereas incubation of erythrocytes with lipid vesicles can increase phospholipid concentrations by incorporation of the phospholipids from the vesicles into cell membranes (8, 56). Normal erythrocytes acti-

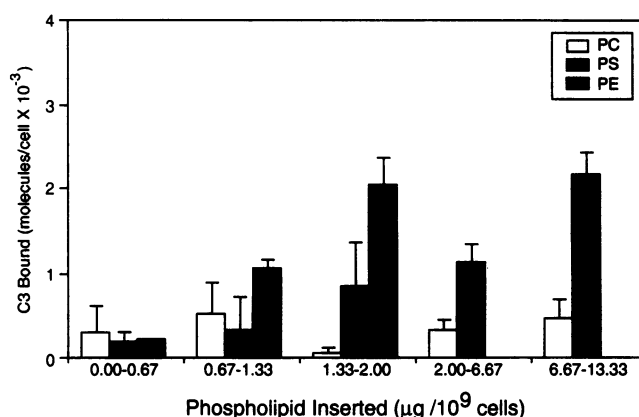


Figure 7. Alternative pathway activation in autologous serum by normal erythrocytes after incubation with PC, PS, or PE vesicles. Erythrocytes from four normal donors were incubated with trace-labeled PC, PS, or PE and incorporation of phospholipids was determined by flow cytometry. Erythrocytes were then incubated with MgEGTA-treated autologous serum. Cells were washed, lysed, and bound C3 was measured by a competitive ELISA. The C3 bound is shown for erythrocytes grouped by the amount of phospholipid inserted. The maximum PE incorporated was $2\text{ }\mu\text{g}/10^9$ cells.

vated complement after either sodium tetrathionate treatment or incubation with PE or PS.

Thiol-oxidizing agents have also been used to study human erythrocyte senescence. It has been reported that treatment with diamide leads to the binding of naturally occurring antibody to aggregated band 3 and that this induces complement activation through the alternative pathway (52). Antibody was not required for complement activation by sickle or tetrathionate-treated erythrocytes, since both showed C3 deposition after incubation with purified alternative pathway components in the absence of antibody. Preabsorption of serum with tetrathionate-treated erythrocytes also did not prevent activation by tetrathionate-treated cells. Since the erythrocytes were separated from serum before deoxygenation or tetrathionate treatment, and antibody binding requires aggregation of membrane proteins, it is unlikely that antibody was already bound to the cells. These results and those using inserted phospholipids indicate that phospholipid changes can induce complement activation in the absence of antibody.

Both PE and PS are exposed on tetrathionate-treated and deoxygenated sickle erythrocytes. Deoxygenation of sickle erythrocytes increases outer leaflet PE from 12 to ~40% of the total membrane PE and PS from 0 to ~20% of the total membrane PS (3–5). The use of labeled individual phospholipids allowed comparison of the effects of PE and PS on complement activation. Although PE was poorly incorporated, probably because of its inability to form bilayered vesicles, an increase of 1.3–2 $\mu\text{g PE}/10^9$ erythrocytes (representing an increase of ~2% of the total PE) in normal erythrocyte membranes resulted in increased complement activation. A dose-dependent increase in C3 deposition was observed for erythrocytes with 1.3–13 $\mu\text{g PS}/10^9$ erythrocytes, but this increase was only significant for erythrocytes containing 6.7–13 $\mu\text{g PS}/10^9$ erythrocytes. This represents an increase of 9–18% of the total membrane PS and is similar to the concentration found on deoxygenated sickle erythrocytes. In contrast, PC insertion did not induce complement activation at the same con-

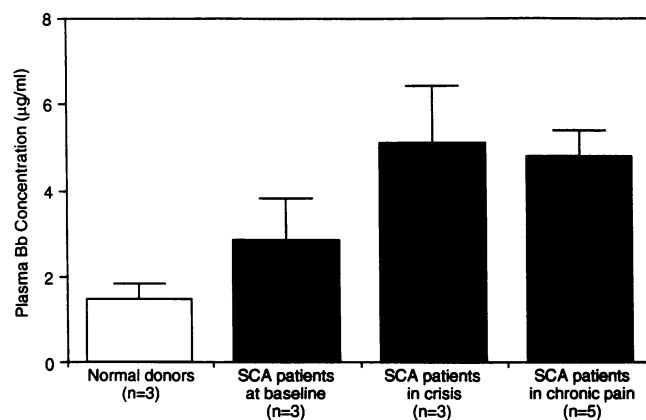


Figure 8. Fragment Bb levels in plasma from normal donors and SCA patients at baseline, in crisis, or in chronic pain. Citrated plasma samples from normal donors or SCA patients were assayed by a commercial ELISA for fragment Bb to measure of *in vivo* alternative pathway activation. Plasma Bb concentrations for SCA patients in crisis are significantly higher than Bb levels of normal donors or SCA patients at baseline ($P = 0.037$). Values for SCA patients in chronic pain are also significantly higher than normal ($P = 0.001$).

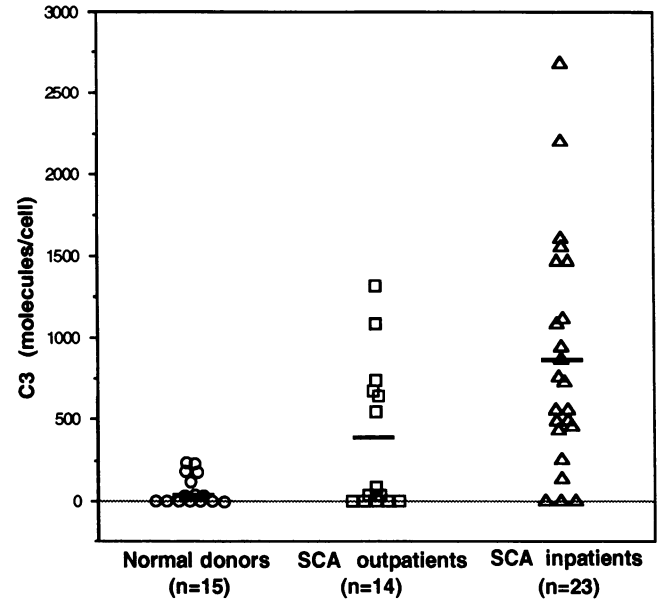


Figure 9. C3 levels on erythrocytes from normal donors, SCA patients seen as outpatients, and hospitalized SCA patients. Erythrocytes were washed, lysed, and tested for C3 by competitive ELISA. Mean values of C3 bound to erythrocytes for 23 hospitalized patients (SCA inpatients) (855 ± 147 molecules/cell) and 14 SCA outpatients (369 ± 122 molecules/cell) were significantly higher than those of 15 normal donors (74 ± 23 molecules/cell).

centrations ($\geq 13 \mu\text{g}/10^9$ erythrocytes). The amount of C3 bound per cell in the phospholipid insertion experiments was lower than that observed with sickle or tetrathionate-treated erythrocytes. However, as noted, the concentrations of PE inserted were also much lower than the amount of PE exposed by the treatments, and possible combined effects of PE and PS were not examined. On the basis of these studies and previous studies using liposomes (21–24), complement activation is apparently more sensitive to PE exposure than to PS exposure. In contrast, PS has been implicated in activation of the coagulation pathway and in binding of erythrocytes to monocytes (2, 7–9).

Complement activation on autologous cells is normally blocked by membrane regulatory proteins. On erythrocytes there are two membrane proteins that regulate C3 convertases, DAF (43) and CR1 (26). DAF is a phosphatidylinositol-anchored membrane protein, which promotes the dissociation of C3 convertases, preventing further complement activation (57). CR1 is a receptor for complement-fixing immune complexes (26). CR1 can also dissociate both C3 convertases and act as a cofactor for the factor I-mediated degradation of C3b and C4b molecules generated by either the classical or alternative pathway. Several additional membrane proteins act on the membrane attack complex to prevent lysis. Since there was no indication of lysis of erythrocytes by the treatments used in these experiments, the functional activities of these regulatory proteins are apparently sufficient to prevent lysis despite the deposition of C3b.

No modification of the function of DAF was observed after incubation of liposomes containing purified DAF with sodium tetrathionate, indicating that the treatment did not directly inactivate DAF. No significant changes in antigenic level or in

the function of DAF and CR1 were detected in sickle or tetrathionate-treated erythrocytes. This is consistent with the low levels of C3 found on erythrocytes isolated from healthy SCA patients before deoxygenation and serum incubation. Increased bound C3 in the form of C3d and C3dg has been reported in conditions of ongoing complement activation associated with reduced CR1 or DAF levels (53). This also indicates that in vitro complement activation by sickle and tetrathionate-treated erythrocytes resulted from the increased appearance of activators rather than decreased function of regulatory proteins. The degree to which complement activation may have been limited by the regulatory proteins was not determined in these experiments. Studies in liposomes in which inserted PE leads to complement activation have demonstrated that this activation is sensitive to inhibition by DAF (44).

Previous studies (15–18) have found evidence of ongoing complement activation in SCA patients. For the most part, no cause of the complement activation was hypothesized, although one study (15) reported a correlation between C3d levels and hemoglobin and suggested that intravascular hemolysis leads to complement activation in these patients. We have demonstrated here an increase in factor Bb concentration in the plasma obtained from three SCA patients experiencing sickle crisis compared with matched baseline values. SCA patients suffering from chronic pain also showed increased plasma Bb compared with normal donors. Since Bb is generated during the formation of the alternative pathway C3 convertase, it is a direct indication of in vivo complement activation during sickle crisis. The plasma Bb levels correlated with increases in fibrin fragment D dimer (a product of fibrinolysis) in identical plasma samples during sickle crisis (Phillips, G., unpublished results). Earlier reports have demonstrated, by using erythrocyte ghosts (54), liposomes (58), or sickle erythrocytes (7), that exposure of membrane PS enhances clotting activities, which may in turn activate the fibrinolytic pathway. The coincident elevation of factor Bb fragment and fragment D dimer suggests that phospholipid redistribution induced in sickle erythrocytes by low oxygen tension leads to the activation of both fibrinolytic and complement cascades, which could recruit additional factors contributing to acute painful sickle crisis.

In addition, hospitalized SCA patients were found to have increased amounts of C3 or C3 fragments bound to erythrocytes in vivo. Exposure of PE and PS on sickle erythrocytes is expected to occur during sickle crisis due to deoxygenation resulting from vasoocclusion. Thus these results are consistent with the hypothesis that altered membrane phospholipids on sickle erythrocytes cause complement activation in vivo. The amount of C3 detected by ELISA on control erythrocytes (74 ± 23 molecules/cell) was similar to a previously reported value of 50 ± 23 molecules/cell (53). The levels of C3 found on erythrocytes from hospitalized SCA patients (855 ± 147 molecules/cell), although higher than controls, are much lower than the mean of $8,673 \pm 7,151$ molecules/cell found on erythrocytes from patients with cold agglutinin disease, an autoantibody-mediated hemolytic anemia.

Complement activation may also contribute to the decreased resistance to bacterial infections in SCA patients. Although we did not find decreased complement in serum from SCA patients studied at baseline, we did find evidence of activation during crisis. In addition, phagocytosis of sickle erythrocytes by macrophages after complement activation (59) or by

direct recognition of exposed PS (8) may overload the phagocytic clearance system, preventing its normal function in the removal of invading microorganisms.

Erythrocyte membrane phospholipid redistribution is not unique to sickle erythrocytes. Increased exposure of PE and PS has been reported in senescent or aged erythrocytes (60), erythrocytes from chronic myeloid leukemia patients (61), and malaria-infected erythrocytes (62). For some of these conditions, there is evidence for complement activation as well (63, 64). Increased PS has been shown to enhance senescent erythrocyte adherence to phagocytes and endothelial cells (2, 8, 9). We have found that human red blood cells aged in vitro can activate the alternative complement pathway resulting in increased C3 deposition (Wang, R., unpublished results). Malaria-infected erythrocytes have increased exposure of PE and PS (62), increased surface C3 binding, and increased phagocytosis by macrophages (59).

In summary, we have presented evidence that altered membrane phospholipid distribution on human erythrocytes is sufficient to initiate complement activation perhaps contributing to opsonization and clearance of abnormal erythrocytes in SCA and other conditions.

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