Raised Neutrophil Phospholipase A₂ Activity and Defective Priming of NADPH Oxidase and Phospholipase A₂ in Sickle Cell Disease

By Elahe Mollapour, J ohn B. Porter, Richard Kaczmarski, David C. Linch, and Pamela J. Roberts

Intermittent painful crises due to vasoocclusion are the major clinical manifestation of sickle cell disease (SCD), but subclinical episodes may also occur. There is sparse evidence for the involvement of neutrophils in the pathophysiology of SCD, but production of cytokines by the damaged endothelium might influence neutrophil function and modulate responses to subsequent cytokine exposure. In addition, the activation of neutrophils in the microcirculation could itself exacerbate vasoocclusion. To test whether neutrophil inflammatory responses were altered in SCD, neutrophil phospholipase A₂ and NADPH oxidase activity in response to in vitro priming by granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor-α (TNF-α) were measured both during and between painful crises. Resting levels of neutrophil phospholipase A₂ activity in steady-state SCD (4.0% ± 0.5% of total cell radioactivity) were raised relative to control values (2.0% ± 0.2%, n = 10, P = .008). There was no defect of agonist-stimulated phospholipase A₂ or NADPH oxidase activity in steady-state SCD; however, the ability of phospholipase A₂ to respond to priming with GM-CSF was attenuated to 63% ± 17% of control values (n = 10, P = .04). Similarly, neutrophil NADPH oxidase activity after priming with GM-CSF and TNF-α was, respectively, 65% ± 11% (n = 7, P = .03) and 57% ± 7% of control (n = 10, P = .007) in steady-state disease, and was further reduced during painful vasoocclusive crises to 34% ± 9% and 25% ± 3% of control for GM-CSF and TNF-α, respectively. These data were not explained by poor splenic function or any racial factor, as normal cytokine responses were seen in splenectomized patients in remission from Hodgkin’s disease and in healthy Afro-Caribbean subjects. Abnormal neutrophil cytokine priming responses were not observed in either patients with rheumatoid arthritis or iron-deficiency anemia. Our findings are indicative of an ongoing inflammatory state in SCD between painful crises involving neutrophil activation and an abnormality of cytokine-regulated neutrophil function, which may compromise the host defenses against certain microorganisms.

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SICKLE CELL DISEASE (SCD) is caused by a point mutation in the β chain of hemoglobin resulting in polymerization of hemoglobin S at low oxygen tensions. This leads to spicule formation, red blood cell (RBC) rigidity, and distortion with consequent vasoocclusive episodes and hemolytic anemia. Oxidative damage occurs to the proteins and lipids of the RBC membrane, with loss of bilayer asymmetry. This disorganization exposes lipids and protein adhesion molecules, and induces the binding of plasma adhesion molecules, thus increasing the tendency for sickled RBC to adhere to endothelium and platelets. Adherence of sickle RBC stimulates endothelial cells to upregulate their adhesion molecules, which accelerates the adhesion cascade. Activation of the endothelial cells results in increased metabolism of endothelial cell phospholipids, with release of eicosanoids and downstream proinflammatory products of both lipoxygenase and cyclooxygenase enzymes. Activated endothelium also releases a wide range of cytokines, including granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-1, IL-3, IL-6, and tumor necrosis factor (TNF), and these have been detected in the plasma of patients with SCD. Sickled RBC also activate platelets, which adhere and release their granule contents, initiating the thrombotic cascade.

Neutrophils may also become activated during this cascade of vasoocclusive events and neutrophil adherence may contribute to vasoocclusion, as well as endothelial cell damage. Activation of endothelial cells and platelets by sickle RBC may affect neutrophil function, and a recent in vitro study demonstrated that neutrophils can be directly activated by sickle RBC. Patients with SCD have an increased tendency to infection, especially with encapsulated organisms, which is due in part to the poor splenic function, but might also be a feature of altered neutrophil function.

Exposure of neutrophils to cytokines in vitro greatly increases the capacity of the phagocyte NADPH oxidase and phospholipase A₂ to respond to activation via cell-surface receptors for chemotactic peptide or IgG—a process referred to as “priming.” Priming of neutrophils by GM-CSF and G-CSF has been demonstrated in vivo. To participate in the host-defense response, neutrophils must adhere to the vascular endothelium in an infective focus, transmigrate into the tissues, be primed by inflammatory cytokines, and then partake in the processes of phagocytosis and killing of microorganisms. This is largely dependent on the activation of the NADPH-dependent oxidase in a respiratory burst, which generates oxygen metabolites and free radicals. Activation of primed neutrophils also results in the stimulation of phospholipase A₂, with the generation of a range of arachidonic acid derivatives, including the leukotrienes, which have potent chemotactic properties and recruit further phagocytes to the inflammatory site. The priming events induced by the inflammatory cytokines are central to these processes. The aim of this study was to look for neutrophil activation and altered neutrophil inflammatory responses in SCD. We therefore examined the activation state of the neutrophil respiratory burst and phospholipase A₂ both in steady-state SCD and during painful vasoocclusive crises, and...
studied the effects of cytokine-mediated priming on these enzymes.

MATERIALS AND METHODS

Patients and Controls

Twenty-three patients from University College London Hospital and the North Middlesex Hospital who were homozygous for the sickle gene (HbSS) were studied: (a) 17 patients (age 17 to 50 years, 12 males) in steady-state between painful vasoocclusive crises were tested on 20 occasions (10 for NADPH oxidase and 10 for phospholipase A₂). The mean interval since the previous crisis that required hospital admission was 40 ± 10 weeks (range, 3 to 132). All of these patients were asymptomatic at the time of study and were receiving prophylactic antibiotic therapy (penicillin V 250 mg twice daily) and folic acid 5 mg daily. None were receiving repeated blood transfusions, opiates analgesics, or hydroxyurea; (b) six hospitalized HbSS patients (age 17 to 31 years, three males) during vasoocclusive crisis treated by prophylactic antibiotics and analgesia. Two of these patients had received a blood transfusion within 6 days of being tested and their levels of HbS were 47% and 24%, respectively, but their neutrophil functional responses were not different from those of the nontransfused patients in crisis. Non-SCD control groups were: (a) three splenectomized patients in long-term remission from Hodgkin's disease (age 35 to 50 years, two males); (b) six healthy Afro-Caribbeans (age 25 to 36 years, two males); (c) six female patients with rheumatoid arthritis (age 41 to 62 years) who were receiving medication (nonsteroidal antiinflammatory drugs [dichlofenac sodium, salazopyrine, or meloxicam, n = 4], gold injections [n = 1], hydroxychloroquine [n = 1]); (d) six female patients with iron-deficiency anemia (age 27 to 60 years); and (e) 41 healthy laboratory personnel (age 24 to 62 years, 14 males, tested on 48 occasions), one of whom was tested each time that blood from the above categories was tested (same-day controls). In all cases, peripheral blood was obtained with the informed consent of the donor.

Neutrophil Function Tests

For assays of phospholipase A₂, venous blood was taken into 2 mmol/L EDTA and neutrophils were purified by centrifugation (1,400 g for 30 minutes at room temperature) through a discontinuous gradient of Histopaque (Sigma Chemical, Poole, Dorset, UK) (densities, 1.077 and 1.119 g/mL). The neutrophil suspension was washed twice in Dulbecco's phosphate-buffered saline (PBS) (without calcium and magnesium; GIBCO-BRL, Paisley, UK) by centrifugation (170g for 7 minutes at room temperature). Purified cells were resuspended to 2 × 10⁶/mL PBS supplemented with 0.9 mmol/L calcium, 0.5 mmol/L magnesium, and 5 mmol/L d-glucose (PBSG). The cellular composition of the final suspension was 92% ± 1% neutrophils, 2.0% ± 0.2% monocytes, 2.7% ± 0.3% lymphocytes, and 3.2% ± 0.4% erythrocytes, as assessed by Leishman's stain. The purity of patients and control neutrophil suspensions were similar. Neutrophils (1 × 10⁶ in 0.5-mL samples) were incubated for 15 minutes with either 1 µmol/L calcium ionophore, A23187 (Sigma), or PBSG diluent, and phospholipase A₂ activity was measured by the release of arachidonate from radiolabeled phospholipid stores, as previously described. For priming experiments, neutrophils were preincubated with either recombinant human (rh)GM-CSF (a kind gift of Behringwerke, Marburg, Germany) at the concentrations indicated in the text or fetal calf serum (FCS; GIBCO-BRL; final concentration, 0.01% vol/vol) for 20 minutes at 37°C before stimulation with ionophore. For H₂O₂ assays, venous blood was anticoagulated with 10 IU preservative-free heparin/mL (Monoparin; CP Pharmaceuticals, Wrexham, Clwyd, UK). Care was taken to ensure that the patients and their same-day controls were venesected within 30 minutes of each other, as the capacity of neutrophils to produce H₂O₂ diminished if blood was stored for more than 90 minutes at room temperature. Aliquots of blood were incubated at 37°C for 15 minutes with 100 µmol/L dichlorodihydrofluorescein diacetate (DCF; Molecular Probes, Eugene, OR) and then for 30 minutes with either rhGM-CSF or rhTNFα (at the doses stated in the text), or FCS diluent (final concentration, 0.01% vol/vol). Stimulation of NADPH oxidase was with either 1 µmol/L formylmethionyl-leucyl-phenylalanine (FMLP, Sigma) or 20 µmol/L phorbol-myristate-acetate (PMA; Sigma) for 15 minutes. Intracellular H₂O₂ production was measured in a whole-blood flow-cytometric assay of the oxidation of DCF, as previously described. H₂O₂ production by resting cells was determined from the mean cell fluorescence (MCF) of the un gated neutrophil population measured on a linear scale. From this distribution, a "positive" gate was set to include 5% of the brightest cells in the control population. H₂O₂ production stimulated by FMLP and PMA was determined as the product of the percentage and MCF of cells entering the preset positive gate. In the cytokine-priming experiments, these MCF values were normalized by expressing the MCF of cytokine-treated cells as a percentage of the MCF of the diluent control. An estimate of the total H₂O₂ production in a given sample was made by multiplying the percentage of positive cells by the normalized MCF, and the data expressed in arbitrary fluorescence units.

Data Analysis

Unless otherwise stated, the data are the mean ± 1 SE of the number of experiments given in the text. Statistical analysis of the data was performed by Wilcoxon’s matched-pairs signed-ranks test.

RESULTS

Phospholipase A₂

Resting levels of neutrophil phospholipase A₂ activity, as measured by the release of arachidonate from radiolabeled phospholipid stores, were twofold greater in patients with steady-state SCD than same-day controls (Fig 1A). The mean resting rate of arachidonate release in neutrophils from steady-state patients was 4.0% ± 0.5% of total cell radioactivity, and in controls was 2.0% ± 0.2% (n = 10, P = .008). Resting levels of phospholipase A₂ activity did not alter when neutrophils were incubated with either 1 or 10 ng/mL of rhGM-CSF and the differential between patient and control values remained significantly different (Fig 1A).

Stimulation of neutrophils with calcium ionophore (A23187) resulted in the activation of phospholipase A₂ above resting levels (Fig 1B). The increment due to calcium-dependent stimulation was determined by subtracting the background values for control samples stimulated with PBSG alone (in the legend to Fig 1). A23187-stimulated arachidonate release was not significantly different in the steady-state patients compared with their respective same-day controls (SCD patients, 2.6% ± 0.5% of total cell radioactivity; controls, 3.9% ± 0.8%; P = .06). Priming of neutrophils with rhGM-CSF (1 and 10 ng/mL) for 20 minutes before stimulation with A23187 resulted in a dose-dependent increase in phospholipase A₂ activity (Fig 1B), which was significantly smaller in neutrophils from patients with steady-state SCD than their respective controls (Fig 1B). The primed phospholipase A₂ responses of SCD patients was only 63% ± 17% of control values (P < .05, n = 10) when 10 ng/mL GM-CSF was used.

NADPH Oxidase

Activation of the neutrophil NADPH-dependent oxidase was measured by a fluorescent assay of H₂O₂ production. There was
no measurable difference in the resting rate of neutrophil H$_2$O$_2$ production between SCD patients in steady-state (MCF of the un gated neutrophil population, 59 ± 10; n = 9) and their respective same-day controls (MCF, 53 ± 8; n = 9; P > .05). Six patients in painful crisis of SCD were also studied, and again the resting values for neutrophil H$_2$O$_2$ production were not different from control (MCF for patients in crisis, 62 ± 6; same-day controls, 60 ± 8; n = 6, P > .05).

Stimulation of neutrophil NADPH oxidase with the chemotactic peptide, FMLP, increased H$_2$O$_2$ production to between two and four times the resting level (Table 1). The mean FMLP responses in both steady-state and crisis SCD patients were greater than control, but the values were not significantly different (in all cases, P > .05). PMA was a more potent agonist than FMLP and stimulated a 30-fold to 80-fold increase in H$_2$O$_2$ production above resting level (Table 1). The PMA responses of neutrophils from patients in the steady-state were the same magnitude as control, but the PMA responses of neutrophils from patients in crisis were on average only 89% of their same-day controls. This small difference was statistically significant (n = 6, P = .014), but unlikely to be of major physiologic importance. Preincubation with varying concentrations of GM-CSF (0 to 10 ng/mL) and TNF-α (0 to 500 U/mL) before stimulation with FMLP caused a dose-dependent enhancement of neutrophil FMLP-stimulated H$_2$O$_2$ responses in both patient and control samples (Fig 2). Neutrophils from SCD patients in steady-state produced significantly less H$_2$O$_2$ after priming with either cytokine than their same-day controls (Fig 2A and B). At the maximal doses of GM-CSF and TNF-α used, H$_2$O$_2$ production was 65% ± 11% (n = 7, P = .03) and 57% ± 7% (n = 10, P = .007) of same-day controls, respectively. Similar experiments with SCD patients in crisis (Fig 2C and D) showed a more marked defect in neutrophil H$_2$O$_2$ production after priming with cytokines. At maximal doses of GM-CSF and TNF-α, H$_2$O$_2$ production was 34% ± 9% (n = 6, P = .03) and 25% ± 3% (n = 6, P = .03) of same-day controls, respectively.

Representative fluorescence distributions of neutrophils from patients and controls obtained from the fluorescence activated cell sorter are shown in Fig 3. In confirmation of previous studies of cytokine-mediated priming of the respiratory burst measured in whole blood, the increase in FMLP-stimulated fluorescence after TNF and GM-CSF priming was due to an increase in both the percentage of positive cells and their MCF. Table 2 shows that in patients with SCD, there were fewer cells recruited into the positive population and the MCF of these positive cells was also lower than that of controls.

**Studies With Control Groups**

**Asplenic controls.** Adult patients with SCD have poor or absent splenic function. To investigate whether this accounted for the defect seen after priming, neutrophil H$_2$O$_2$ production and cytokine priming were measured in three patients in
long-term remission from Hodgkin’s disease who had been splenectomized many years previously. No defect in cytokine-mediated priming was observed compared with nonsplenectomized controls tested on the same day. The total H₂O₂ production for patients expressed as percentage of the value for the same-day control was 104% ± 6% and 90% ± 5% (n = 3, P > .05), respectively, for samples primed with 1 and 10 ng/mL GM-CSF; and 101% ± 19% and 103% ± 17% (n = 3, P > .05), respectively, for samples primed with 50 and 500 U/mL TNF-α.

Ethnic controls. All but two of the SCD patients studied were of African or Afro-Caribbean descent, whereas the same-day controls were non–Afro-Caribbeans. To examine whether any racial factor may have contributed to the results of the priming experiments, blood from six healthy non-SCD Afro-Caribbeans was studied in parallel with samples from healthy non–Afro-Caribbeans, and no significant differences were seen between the values for these two groups in their priming response to either GM-CSF or TNF-α. The total H₂O₂ production for Afro-Caribbeans expressed as the percentage of the value for non–Afro-Caribbean controls was 105% ± 27% and 100% ± 24% (n = 6, P > .05), respectively, for samples primed with 1 and 10 ng/mL GM-CSF; and 104% ± 17% and 108% ± 23% (n = 6, P > .05), respectively, for samples primed with 50 and 100 U/mL TNF-α.

Patients with arthritis. Six patients with seropositive erosive rheumatoid arthritis, classified according to the American Rheumatism Association criteria, were studied to determine whether the defect in cytokine-mediated priming could be observed in patients with ongoing inflammation. They were
in Fig 4C and D, there was no significant difference in nancy, as this might affect their neutrophil responses. As shown Iron-deficient patients were selected who were not infected, or state SCD and 90^6 value for these patients was 76 iron-deficiency anemia were studied. The mean hemoglobin a or TNF-P, between the patient and control groups (data not shown) and, as

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Anemic controls. To investigate whether the priming defect was associated with the anemia of SCD, six females with iron-deficiency anemia were studied. The mean hemoglobin value for these patients was 76 ± 5 g/L (mean ± 1 SE), compared with 81 ± 13 g/L (n = 8) for patients with steady-state SCD and 90 ± 16 g/L (n = 6) for SCD patients in crisis. Iron-deficient patients were selected who were not infected, or recovering from surgery, or suffering from any form of malignancy, as this might affect their neutrophil responses. As shown in Fig 4C and D, there was no significant difference in FMLP-stimulated neutrophil H2O2 production between patients with iron-deficiency anemia and their sex-matched controls either with or without prior priming by TNF-α or GM-CSF (in all analyses, P > .05, n = 6).

DISCUSSION

Activation of neutrophils as evidenced by enhanced neutrophil aggregation was previously noted during episodes of vasoocclusive crisis in SCD, but there is little published evidence of neutrophil activation during the steady-state. If there is ongoing interaction between damaged endothelium and activated neutrophils in the steady-state, it is conceivable that this may contribute to the initiation of vasoocclusive crises. Agents that decrease such interactions might therefore be of value to reduce the frequency of painful crises. This study shows that neutrophil phospholipase A2 activity in steady-state SCD is increased approximately twofold compared with control, demonstrating that neutrophils are activated in steady-state disease. The mechanism of this activation is unclear at present. A recent study showed that the neutrophil NADPH oxidase could be activated by contact with sickled RBC in vitro, and neutrophil phospholipase A2 may also be activated in this way. However, we found no evidence for parallel basal activation of NADPH oxidase activity of neutrophils in SCD when tested in the whole-blood milieu. Release of phospholipase A2 from activated neutrophils might contribute to the threefold increase above control in the plasma levels of the secreted isoform of phospholipase A2 that were recently detected in patients with steady-state SCD.

Our study also demonstrates that neutrophils from patients in the steady-state of SCD have reduced NADPH oxidase and phospholipase A2 responses to the agonists FMLP and calcium ionophore after in vitro priming with the cytokines GM-CSF and TNF-α. The amounts of H2O2 and arachidonate produced were approximately 50% of that produced by control cells and, in addition, the defect in oxidase activity was more severe in

![Graphs](https://www.bloodjournal.org)
patients in crisis. One consequence of suboptimal oxygen radical production after priming would be the failure to kill microorganisms that require high concentrations of \( \text{H}_2\text{O}_2 \) for their destruction. Such organisms are likely to be those that have developed protective mechanisms against the phagocyte respiratory burst, such as encapsulation, catalase production, or oxidase inhibition. Thus, the neutrophil defect could potentially exacerbate the infective risks with \textit{Streptococcus pneumoniae} and \textit{Haemophilus influenzae} that accompany the hyposplenic state in sickle cell syndromes.

Priming of the NADPH oxidase by GM-CSF and endotoxin has been attributed to increased production of arachidonate\textsuperscript{10,19}; thus, the inability of SCD neutrophils to fully upregulate NADPH oxidase activity could be a sequela of the suboptimal activation of phospholipase A\(_2\) by agonist after cytokine-mediated priming. Unprimed neutrophils from steady-state SCD patients produced equivalent amounts of \( \text{H}_2\text{O}_2 \) as control cells in response to stimulation with both FMLP and the more potent stimulus, PMA (elicits maximal production of \( \text{H}_2\text{O}_2 \) in control cells), suggesting that the deficit in priming was not explained by an intrinsic problem with the NADPH oxidase itself. This is in accord with previous studies demonstrating a normal respiratory burst in unprimed phagocytes from patients with SCD.\textsuperscript{20,22} Assays of phospholipase A\(_2\) used washed and purified neutrophils, unlike the NADPH oxidase assay, which was performed in whole blood, demonstrating that the reduced responses to cytokine-mediated priming were an inherent cellular defect.

Spicule formation in sickled RBC with concomitant destabilization of membrane organization promotes adherence of RBC to endothelium resulting in stimulation of endothelial cells. These cells when activated can secrete a wide range of biologic response modifiers, which may contribute to the increased levels of GM-CSF, IL-1, IL-3, IL-6, and TNF-\( \alpha \) that have been detected in the peripheral blood of some SCD patients.\textsuperscript{3,4,22,23} This raises the possibility that the defective in vitro cytokine-mediated priming of neutrophils might be due to prior exposure to cytokines in the circulation. This would leave the cells refractory to further priming, either because of downregulation of cytokine receptors or postreceptor signal transduction mechanisms. Previous work has shown that neutrophils primed in vivo by infusion of GM-CSF are not responsive to subsequent in vitro priming with GM-CSF, and after a period of 2 hours have a priming capacity that is reduced to approximately 50% of preinfusion values,\textsuperscript{13,14} confirming that this is a possible mechanism.

Surprisingly, there was little evidence of recent or sustained in vivo priming of neutrophils as markedly elevated responses to agonist were not observed in either the NADPH oxidase or PLA\(_2\) assays when cells were stimulated in the absence of in vitro cytokines. This might have been expected if cells had been recently primed in vivo, as demonstrated by our previous studies,\textsuperscript{13,14} but there are two explanations why evidence of recent priming was not observed. First, activation of neutrophils by interaction with damaged endothelium may only be transient. For example, the primed state of the neutrophil NADPH oxidase can be short-lived when induced by platelet-activating factor (1 to 2 hours)\textsuperscript{24} or by cytokines such as IL-8 (30 minutes) and TNF-\( \alpha \) (1 hour), whereas the response to GM-CSF can last for several hours.\textsuperscript{25} It is possible that there had been in vivo priming with such a transient priming agent and we were testing the cells during a postpriming refractory period. Second, inflammatory events in SCD may produce a range of activated states in the neutrophil. Fully activated phagocytes are lost from the circulation, as evidenced by a reduction in levels of circulating neutrophils after in vivo infusions of cytokines\textsuperscript{26} due to margination or migration into the tissues.\textsuperscript{27} In our study, neutrophils primed in vivo may similarly have left the circulating pool, leaving the less activated cells to be collected from the peripheral blood for in vitro assays.

In an attempt to further understand the mechanism of altered neutrophil function in SCD, we studied four groups of people who did not have SCD. Racial differences in neutrophil function and poor splenic function were excluded as mechanisms, as no defects in neutrophil \( \text{H}_2\text{O}_2 \) production were seen in either healthy Afro-Caribbean subjects or splenectomized patients when tested against control subjects. The effects we observed in SCD were also not reproduced in a group of patients with active rheumatoid arthritis. Our study appears to be the first to test neutrophil function in whole blood from patients with arthritis, whereas previous studies using purified cells or diluted whole blood have produced contradictory results, some showing evidence of activated\textsuperscript{28-30} or defective\textsuperscript{31,32} neutrophils in the circulation with reduced responses after cytokine-mediated priming,\textsuperscript{29,31} with others showing no difference from control.\textsuperscript{33,34} Our findings with rheumatoid arthritis patients emphasize that the defective priming seen in SCD is likely to be specifically associated with events in the circulation, rather than being a general feature of inflammatory disorders. Finally, we investigated whether the priming defect could be attributed to the marked anemia of the patients with SCD, who had hemoglobin levels of 80 to 90 g/L. However, we could find no evidence for reduced \( \text{H}_2\text{O}_2 \) production or cytokine-mediated priming in a series of patients with iron-deficiency anemia whose mean hemoglobin level was 76 ± 5 g/L (\( n = 6 \)).

In conclusion, we have shown definitive evidence of neutrophil activation with increased phospholipase \( \text{A}_2 \) activity in steady-state SCD, which may contribute to the triggering of vasoocclusive crises. This observation may provide a rationale for beneficial therapeutic intervention with antiinflammatory drugs. In addition, we demonstrated that neutrophils in the peripheral blood of SCD patients have a limited capacity to respond to priming with cytokines, and this may contribute to the susceptibility of these patients to infection.

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