High Levels of Neopterin and Interleukin-3 in Sickle Cell Disease Patients

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Sickle cell disease (SCD) is recognized as a chronic inflammatory condition. Cytokines are released in response to stress or pathological situations, and influence hematopoiesis. The aim of this study was to evaluate interleukin-3 (IL-3), interferon- γ (IFN- γ), and neopterin (NP) levels in steady-state patients with sickle cell anemia (SS) ($n = 35$) and SC hemoglobinopathy ($n = 15$) in order to verify the possible action of those cytokines and NP on iron metabolism and hematopoiesis. Serum IL-3 concentration was higher in SS and SC groups than in controls, whereas IFN- γ levels did not differ among groups. SS patients presenting hemoglobin fetal (HbF) $\geq 8.5\%$ had significantly higher IL-3 levels than those with HbF $< 8.5\%$ ($P = 0.0338$). No correlation was observed among inflammatory and

iron metabolism parameters. Significant correlations were observed between IL-3 and Hb levels ($r = 0.4633$, $P = 0.0457$), and IL-3 and HbF levels ($r = 0.6011$, $P = 0.0065$). A negative correlation was observed between IL-3 and reticulocyte counting ($r = -0.4632$, $P = 0.0457$) only in the SS group. NP levels were significantly higher in the SS and SC groups than in controls, but did not differ between SS and SC. No correlation was observed between NP and iron metabolism parameters. These data suggest that IL-3 stimulates hematopoiesis, and that SS patients, even in steady state, have macrophage/monocyte activation (represented by high levels of NP) that probably contributes to their chronic inflammatory condition. *J. Clin. Lab. Anal.* 20:75–79, 2006. © 2006 Wiley-Liss, Inc.

Key words: cytokines; hemoglobinopathy; hemoglobin S; sickle cell anemia; hematopoiesis; fetal hemoglobin; steady state

INTRODUCTION

Sickle cell disease (SCD) manifestations are due to the substitution of a nucleotide base in codon 6 of the beta-globin gene. Red cells containing Hb S become rigid and are unable to pass through small arteries and capillaries. Vasoocclusive crises associated with severe infections are frequent, and currently both immune and inflammatory processes are considered to be closely associated with clinical manifestations in SCD patients (1–3). White blood cells contribute to vasoocclusion, stimulating the vascular endothelium to increase the expression of ligands for adhesion molecules. Obstruction of the lumen causes tissue damage and inflammatory reactions (4).

Cytokines are released in response to stress or pathological conditions, and influence hematopoiesis by enhancing or inhibiting cellular differentiation and proliferation. For instance, stem cell factor (SCF) and interleukins 3 and 7 (IL-3 and IL-7) stimulate hemato-

poiesis, and tumor necrosis factor α (TNF- α) and interferon γ (IFN- γ) are inhibitory factors of erythropoiesis (5). IFN- γ released during an infectious or inflammatory process acts negatively on transferrin receptor synthesis, stimulates iron capture from circulation, and enhances mRNA ferritin expression. The reduced disposable iron leads to impaired hemoglobinization and anemia (6).

Neopterin (NP) is a metabolite of guanosine triphosphate and is produced by monocytes/macrophages stimulated by IFN- γ (7). It is considered a useful

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marker of immunological activation, and NP measurement has been used to predict transplant rejection and monitor infectious autoimmune disorders and renal diseases (reviewed in Ref. 8).

There is a decreasing in blood iron content during immune response. Supposed mechanisms involved in hypoferraemia, such as iron request for proliferation and differentiation of immune cells, and ferritin production stimulated by TNF- α , IL-1, and IL-6, lead to a reduction in circulating iron. In addition, lower intracellular iron concentration increases the release of NP by monocytic cells stimulated by IFN- γ (9). NP has an indirect inhibitory effect on erythropoiesis by decreasing erythropoietin mRNA levels (10).

Increased NP concentrations are observed in infectious diseases, malignancies, and autoimmune diseases (reviewed in Ref. 8). Previous studies showed an inverse correlation between NP levels and hemoglobin, and a positive correlation between NP and ferritin levels in patients with chronic diseases with accompanying anemia (11).

There is evidence that a chronic inflammation condition exists in SCD patients even in steady state (1). The aim of this study was to evaluate the cytokine and NP levels in SCD patients and investigate the possible relationship between the inflammatory process and erythropoiesis in sickle cell anemia (SS) and SC hemoglobinopathy patients.

MATERIALS AND METHODS

Fifty SCD patients (35 SS homozygous and 15 SC double heterozygous) who were seen at the Center of Hemotherapy and Hematology, State University of Campinas, SP, Brazil, in steady state (no acute complications) were studied. They ranged in age from 17 to 54 years (mean = 34 years) in the SS group, and from 14 to 48 years (mean = 30 years) in the SC group. Twenty-six healthy individuals (21–50 years old, mean = 35 years), without clinical and laboratory data concerning anemia or infection/inflammatory disease were selected as the control group. There was no significant difference in age and gender distribution among the studied groups (Kruskal-Wallis test). None of the patients were receiving hydroxyurea treatment or had received a blood transfusion during the 3 months prior to sample collection.

Informed consent was obtained from all individuals, consistent with the guidelines of the local ethics committee.

Laboratory Studies

Blood samples were collected by venipuncture. Serum was stored at -20°C until assayed. Complete blood

counts (CBCs) and reticulocyte counts were determined with the use of the Sysmex XE-2100 (Sysmex, Kobe, Japan). HbF measurement was obtained by high-performance liquid chromatography (HPLC, Bio-Rad Variant; Bio-Rad Laboratories, Hercules, CA). Iron metabolism was assessed by serum iron (SI), total iron binding capacity (TIBC; Synermed International, Westfield, IN), and serum ferritin (SF; Elecsys; Roche Diagnostics, Germany) levels.

– For the erythropoiesis evaluation, soluble receptor of transferrin (sTfR) was detected by nephelometry (Dade Behring, Marburg, Germany), and erythropoietin (EPO) was detected by immunometric assay (Immulite EPO; DPC, Los Angeles, CA).

– Serum levels of cytokines and NP were measured using commercially available immunoassays kits: using IL-3 (R&D Systems, Inc., Minneapolis, MN), IFN- γ (Pharmingen, San Diego, CA), and NP (IBL Immuno-Biological Laboratories, Hamburg, Germany). The kits used to measure IL-3 and IFN- γ employed a high-sensitivity technique.

Statistical Analysis

The Mann-Whitney test was used to compare two independent groups, and Kruskal-Wallis was used to compared three or more groups. Spearman's rank correlation was used in the correlation analyses. The data are presented as the median and range. Statistically significant differences were defined as comparisons resulting in $P < 0.05$. All of the calculations were made in the SAS System for Windows, version 8.2 (SAS Institute Inc., Cary, NC).

RESULTS

Our results showed that, as expected, anemia and hemolysis degrees were more severe in SS patients than in patients with SC hemoglobinopathy, according to Hb and reticulocyte values. Erythropoietic activity was evaluated by sTfR measurement and it was higher in SS patients than in the SC group, and both were higher compared to the control group (Table 1). A positive correlation between sTfR and reticulocyte counts was observed in SS patients ($r = 0.4341$, $P = 0.0091$). Regarding iron status, SS and SC patients showed SI and TIBC levels significantly lower than the control group, but serum ferritin values were higher in both groups compared to control individuals (Table 1).

Nineteen SS patients (54.2%), seven SC patients (46.6%), and 12 control individuals (46.1%) had detectable levels of serum IL-3. In those individuals

TABLE 1. Hematological and iron status parameters in SS and SC patient groups, and control group (median and range)

Parameters	Control group (C) (n = 26)	SS group (n = 35)	SC group (n = 15)
Hb (g/dL)	14.0 (12.0–17.3)	8.2 ^{*,**} (5.4–14.1)	12.0 ^{*,**} (9.5–14.1)
Hb F (%)	1.05 (0.3–1.3)	6.3 ^{*,**} (0.7–22.0)	1.5 ^{*,**} (0.3–6.1)
RTC ($\times 10^9$ /L)	43.2 (18.9–93.1)	246.8 ^{*,**} (131.5–606.2)	108.9 ^{*,**} (63.9–201.3)
WBC ($\times 10^9$ /L)	6.6 (3.3–11.5)	9.2 ^{*,**} (4.0–15.1)	7.9 ^{*,**} (3.1–12.6)
sTfR (mg/L)	1.5 (0.9–3.1)	9.84 ^{*,**} (3.19–19.5)	3.58 ^{*,**} (2.25–6.01)
SI (μ g/dL)	84.0 (36.0–158.0)	58.0 [*] (25.0–145.0)	63.0 [*] (30.0–97.0)
TIBC (μ g/dL)	372.5 (291.0–493.0)	236.0 ^{*,**} (87.0–390.0)	329.0 ^{*,**} (192.0–449.0)
SF (ng/mL)	51.9 (13.0–252.6)	118.7 [*] (27.5–2385.0)	201.3 [*] (16.6–1773.0)
EPO (μ m/mL)	8.3 (4.2–17.2)	64.1 ^{*,**} (16.2–274.0)	27.5 ^{*,**} (12.2–5.3)

Hb, hemoglobin; Hb F, fetal hemoglobin; RTC, reticulocyte count; WBC, white blood cells; sTfR, soluble transferrin receptor; SI, serum iron; TIBC, total iron-binding capacity; SF, serum ferritin; EPO, erythropoietin.

* $P < 0.05 \times C$.

** $P < 0.05$ SS $\times$ SC groups.

serum IL-3 concentrations were statistically higher in the SS and SC groups compared to the control group ($P = 0.0350$ and $P = 0.0280$, respectively), but not between the patient groups. NP levels were significantly increased in the SS and SC groups compared to controls ($P < 0.0001$ and $P = 0.0336$, respectively). Between SS and SC patients there was no difference (Table 2). Positive significant correlations between IL-3 and Hb ($r = 0.4633$, $P = 0.0457$), and IL-3 and HbF ($r = 0.6011$, $P = 0.0065$) were observed, and a negative correlation between IL-3 and RTC ($r = -0.4632$, $P = 0.0457$) was observed in the SS group but not in the SC and control groups.

Twelve healthy controls (46.1%), 19 SS patients (54.2%), and seven SC patients (46.6%) had detectable levels of IFN- γ , and the difference among groups was not significant (Table 2).

When SS patients were subdivided according to Hb F levels, 21 showed Hb F $< 8.5\%$ (LHbFSS) and 14 had Hb F $\leq 8.5\%$ (HHbFSS). Total Hb levels were lower in LHbFSS patients than in HHbFSS patients ($P = 0.0116$). IL-3 concentrations were significantly higher in HHbFSS patients ($P = 0.0338$) than in LHbFSS patients. IFN- γ , NP, and sTfR levels did not differ between the groups (Table 3).

TABLE 2. Cytokines and NP levels in control and patient groups (median and range)

	Control group (C)	SS group	SC group	P value
IL-3 (pg/mL)	17.3 (n = 12) (3.10–116.4)	46.6 (n = 19) (11.5–296.0)	67.3 (n = 7) (12.2–145.0)	0.0350 (SS $\times$ C) 0.0280 (SC $\times$ C)
IFN- γ (pg/mL)	2.0 (n = 12) (1.1–6.0)	2.5 (n = 19) (1.0–22.0)	2.0 (n = 7) (1.0–3.6)	NS
NP (nmol/L)	3.95 (n = 26) (2.2–7.5)	6.90 (n = 35) (2.8–33.0)	5.9 (n = 15) (2.5–8.9)	< 0.0001 (SS $\times$ C) 0.0336 (SC $\times$ C)

NP, neopterin; IL-3, interleukin 3; IFN- γ , interferon γ ; NS, not significant.

TABLE 3. Hb, sTfR, IL-3, IFN- γ , and NP levels in SS patient subgroups according to HbF percentage (median and range)

	HHbFSS (n = 14)	LHbFSS (n = 21)	P value
Hb (g/dL)	8.6 (6.5–14.1)	7.1 (5.4–10.2)	0.0116
NP (nmol/L)	6.2 (3.1–14.0)	7.0 (2.8–33.0)	0.3722
sTfR (mg/L)	8.74 (3.19–19.5)	10.2 (6.93–15.6)	0.3206
IL-3 (pg/mL)	74.7 (n = 9) (11.5–296.0)	32.6 (n = 10) (14.4–103.10)	0.0338
IFN- γ (pg/mL)	2.0 (n = 6) (1.1–16.7)	2.7 (n = 13) (1.6–22.0)	0.40470

Hb, hemoglobin; NP, neopterin; sTfR, soluble transferrin receptor; IL-3, interleukin 3; IFN- γ , interferon γ ; HHbFSS, HbF $\geq 8.5\%$; LHbFSS, Hb F $< 8.5\%$.

DISCUSSION

The role of inflammation in the physiopathology of SCD has been the target of recent investigations (3,4,12). The rigid sickle erythrocyte interacts with leukocytes, platelets, plasma proteins, and blood vessel walls, reducing the blood flow and causing infarcts in different organs. Several molecules mediate leukocyte adhesion to the vascular endothelium, such as intercellular adhesion molecule (ICAM), platelet endothelial cell adhesion molecule-1 (PECAM-1) and L-selectin. Vasoocclusion causes ischemia, infarction, and pain. Vasoactive substances, interleukins, and highly reactive oxygen radicals are released from leukocytes, causing oxidative damage and increasing the inflammatory reaction (4).

IFN- γ participation in inflammatory process in SCD is not completely elucidated. Raghupathy et al. (13) reported that IFN- γ plasma levels are similar in SS patients and controls, and Taylor et al. (14) observed

lower IFN- γ levels in steady- and crisis-state SCD patients. In another study elevated levels of IFN- γ were observed in steady-state SCD patients compared to normal subjects, but there was no difference between steady- and crisis-state groups (15). In our study patients with SCD presented IFN- γ levels similar to those in controls, in agreement with Raghupathy et al. (13). The great diversity of IFN- γ level determinations in clinical studies is probably due to IFN- γ instability and to the technical limitations of plasma detection by current assays.

Proinflammatory cytokine IFN- γ seems to affect iron homeostasis by different mechanisms: IFN- γ depresses TfR expression by transcriptional and post-transcriptional mechanisms, induces ferritin mRNA expression in macrophages. In contrast, it represses ferritin mRNA translation by nitric oxide action on IRP-IRE binding within the 5' untranslated mRNA region (reviewed in Ref. 6). According to our results, the lack of correlation between IFN- γ levels and iron parameters in SS and SC patients with detectable serum IFN- γ levels suggests that high levels of ferritin in SCD patients are due to the hemolysis process, which supplants the cytokine action. However, the exact role of IFN- γ in hematopoiesis in SCD requires further studies for better elucidation.

Interestingly, values of NP, a product of macrophage/monocyte activation by IFN- γ , were significantly higher in SS patients. NP measurement has an advantage over other immune system mediators because NP is biologically stable and has been studied as a marker associated with cell-mediated immunity in infectious, malignant, autoimmune, and chronic inflammatory diseases (reviewed in Ref. 8). NP plays physiological roles in inducing apoptosis (16) and enhancing the effect of hydrogen peroxide and chloramine and their toxicity against microorganisms (17).

No correlation between circulating IFN- γ and NP levels was found in our study, in contrast to previous findings in infectious diseases and allograft recipients. NP measurement appears to be superior to IFN- γ because it may reflect the effect of the synergism between other cytokines and IFN- γ on the macrophage population. In addition, NP is biologically stable and easily quantified in human body fluids (reviewed in Ref. 9).

An association between increased NP levels and abnormalities of the hematopoietic systems has been described in patients with malignant and infectious diseases. An inverse correlation between Hb and NP levels was also observed, and NP was associated with abnormalities of iron metabolism (18,19). In our study no correlation between Hb and NP levels, or among NP and several iron parameters was observed, suggesting

that NP does not interfere in erythropoiesis and iron metabolism. Higher NP levels in SS patients are probably due to the chronic inflammatory process. It has been demonstrated that during intravascular hemolysis, the physical separation of hemoglobin from endothelium is disrupted, resulting in efficient nitric oxide scavenging and endothelial dysfunction (20). Activation of macrophages during vascular injury in SCD may lead to release of NP into the blood stream, as demonstrated in an acute episode of cerebral ischemia (21).

Croizat and Nagel (22) studied the participation of circulating cytokines in erythropoiesis in SCD. Stem cell factor (SCF) and granulocyte-macrophage colony stimulating factor (GM-CSF) were higher in LHbFSS than in HHbFSS, and transforming growth factor- β (TGF- β), an inhibitor cell growth, was lower in patients with low levels of HbF, indicating a permanent state of overall increased hematopoiesis in LHbFSS patients in response to higher erythropoiesis stress.

IL-3 is a multilineage stimulator that, in addition to acting with other growth factors, interacts with EPO to stimulate primitive erythroid stem cell (23). The stimulatory effect of IL-3 on erythropoiesis was demonstrated in our study, since IL-3 levels were significantly higher in SS and SC patients compared to controls. IL-3 control of hematopoiesis was determined to be more consistent by comparing IL-3 values in SS subgroups with different levels of HbF: elevated IL-3 concentrations were observed in HHbFSS compared to LHbFSS, in accordance with previously reported results. Studies have shown that levels of IL-3 in SS patients are specifically linked to the degree of hematopoietic stress, which is lower in HHbFSS patients due to the inhibitory effect of HbF on the polymerization of deoxy HbS (22,24).

The lack of correlation between EPO and Hb, and between EPO and HbF may be a consequence of a complex association of variables involved in anemia in SCD, such as renal damage, fault in folic acid supplement and presence of infection process. All of those variables may contribute to a relative reduction of EPO levels for the degree of anemia (25).

In conclusion, this study demonstrates the influence of IL-3, IFN- γ , and NP on iron metabolism in SCD patients. The observed correlation between interleukin and Hb/HbF levels suggests that IL-3 has a positive action on hematopoiesis in SCD patients. As far as we know, this is the first study to evaluate NP in SCD. Our data indicate that SS patients, even in steady state, present a macrophage/monocyte activation, represented by high levels of NP, that probably contributes to the chronic inflammatory condition of SS patients.

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REFERENCES

- Chies JAB, Nardi NB. Sickle cell disease: a chronic inflammatory condition. *Med Hypoth* 2001;57:46–50.
- Platt OS. Sickle cell anemia as an inflammatory disease. *J Clin Invest* 2000;106:337–338.
- Pathare A, Kindi AS, Daar K, Dennilson D. Cytokines in sickle cell disease. *Hematology* 2003;8:329–337.
- Okpala I. The intriguing contribution of white blood cells to sickle cell disease—a red cell disorder. *Blood Rev* 2004;18:65–73.
- Abbas AK, Lichtman AH, Pober JS. editors. *Cellular and molecular immunology*. Philadelphia: WB Saunders Co., 1994.
- Weiss G. Iron and anemia of chronic disease. *Kidney Int* 1999;55: S12–S17.
- Huber CH, Batchelor JR, Fuchs D, et al. Immune response-associated production of neopterin. *J Exp Med* 1984;160:310–316.
- Berdowska A, Zwirska-Korczala K. Neopterin measurement in clinical diagnosis. *J Clin Pharm Ther* 2001;26:318–329.
- Fuchs D, Weiss G, Wachter H. Neopterin, biochemistry and clinical use as a marker for cellular immune reactions. *Int Arch Allergy Immunol* 1993;101:1–6.
- Pagel H, Fandrey J, Schobesberger W, Fuchs D, Jelkmann W. Effects of neopterin and 7,8-dihydroneopterin on hypoxia-induced renal erythropoietin production. *Eur J Haematol* 1999;62:341–345.
- Weiss G, Widner B, Zoller H, Schobesberger W, Fuchs D. Immune response and iron metabolism. *Br J Anaesth* 1998;81:6–9.
- Kaul DK, Hebbel RP. Hypoxia/reoxygenation causes inflammatory response in transgenic sickle mice but not in normal mice. *J Clin Invest* 2000;106:411–420.
- Raghupathy R, Haider MZ, Azizieh F, Abdelsalam R, D'Souza TM, Adekile AD. Th1 and Th2 cytokine profiles in sickle cell disease. *Acta Haematol* 2000;103:197–202.
- Taylor SC, Shacks SJ, Villicana SM, Olivares J, Dinkins GA. Interferon production in sickle cell disease. *Lymphokine Res* 1990; 9:415–423.
- Pathare A, Kindi AS, Alnaqdy AA, Daar S, Knox-Macaulay H, Dennison D. Cytokine profile of sickle cell disease in Oman. *Am J Hematol* 2004;77:323–328.
- Oetl K, Wirleitner B, Baier-Bitterlich G, Grammer T, Fuchs D, Reibnegger G. Formation of oxygen radicals in solutions of 7,8-dihydroneopterin. *Biochem Biophys Res Commun* 1999;264: 262–267.
- Weiss G, Fuchs D, Hausen A, et al. Neopterin modulates toxicity mediated by reactive oxygen and chloride species. *FEBS Lett* 1993; 321:89–92.
- Denz H, Hubber DH, Landmann R, Orth B, Wachter H, Fuchs D. Association between the activation of macrophages, changes of iron metabolism and the degree of anaemia in patients with malignant disorders. *Eur J Haematol* 1992;48:244–248.
- Fuchs D, Reibnegger G, Werner ER, Vinazer H, Wachter H. Low haemoglobin in haemophilia children is associated with chronic immune activation. *Acta Haematol* 1991;85:62–65.
- Reiter CD, Wang X, Tanus-Santos JE, et al. Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease. *Nat Med* 2002;8:1383–1389.
- Anwaar I, Gottsäter A, Lindgärde F, Mattiasson I. Increasing plasma neopterin and persistent plasma endothelin during follow-up after acute cerebral ischemia. *Angiology* 1999;50:1–8.
- Croizat H, Nagel RL. Circulating cytokines response and the level of erythropoiesis in sickle cell anemia. *Am J Hematol* 1999;60: 105–115.
- Quesenberry PJ, Colvin GA. Hematopoietic stem cells, progenitor cells, and cytokines. In: Beutler E, Lichtman MA, Coller BS, Kipps TJ, Seligsohn U, editors. *Hematology*. New York: McGraw-Hill Medical Publishing Division, 2001. p 153–174.
- Croizat H. Circulating cytokines in sickle cell patients during steady state. *Br J Haematol* 1994;87:592–597.
- Sherwood JB, Goldwasser E, Chilcote R, Carmichael LD, Nagel RL. Sickle cell anemia patients have low erythropoietic levels for their degree of anemia. *Blood* 1986;67:46–49.