Original Research

TNF-α, IFN-γ, IL-10, and IL-4 levels were elevated in a murine model of human sickle cell anemia maintained on a high protein/calorie diet

Hyacinth I Hyacinth[1,2], Patrice L Capers[2], David R Archer[3] and Jacqueline M Hibbert[2]

[1]Stroke Center, Department of Neuroscience, Medical University of South Carolina, 19 Hagood Avenue, Suite 501, Charleston, SC 29407, USA; [2]Department of Microbiology, Biochemistry and Immunology, Morehouse School of Medicine, Atlanta, GA 30310, USA; [3]Aflac Cancer Center, department of Hematology and Bone Marrow Transplant, Emory University, NE Atlanta, GA 30322, USA
Corresponding author: Hyacinth I Hyacinth. Email: hyacinth@musc.edu

Abstract

Increased frequency and risk of infection is one of the well described complications of sickle cell anemia (SCA). Dietary supplementation in children with SCA and growth retardation improved growth and decreased incidence of infection. We investigated the impact of a high protein diet on weight gain, hematological profile, and immune cytokine levels in the Berkeley model of SCA, 16 of which were randomized to either regular mouse diet with 20% of calories from protein ($n = 8$) or a test feed with 35% of calories from protein ($n = 8$). Control mice (C57BL/6, $n = 16$) were correspondingly randomized, and were all feed *ad libitum* for three months with actual intake estimated by subtracting the weight of gnaw waste from that of the feed given. Blood was collected at sacrifice by cardiac puncture and plasma levels of T helper cell 1 (TH1) and TH2 associated cytokines were measured using a multiplex antibody immobilized bead assay. SCA mice receiving the 35% protein diet had modest improvements in weight, red blood cell count, and hemoglobin level, with a slight decrease in reticulocyte count compared with SCA mice on the regular mouse diet. Furthermore, they also had significantly higher plasma levels of cytokines tumor necrosis factor (TNF)-α ($P = 0.02$), interferon (IFN)-γ ($P = 0.01$), interleukin 10 (IL-10; $P = 0.02$), and IL-4 ($P = 0.02$) compared with those that received the 20% protein diet. We conclude that providing additional protein calories to transgenic SCA mice increased the plasma levels of acute inflammatory cytokines associated with immune response to infection, which might partly explain decreased episodes of infection observed among supplemented children with SCA.

Keywords: Nutrition, sickle cell disease, cytokines, infection, immunity

***Experimental Biology and Medicine* 2014; 239: 65–70. DOI: 10.1177/1535370213508357**

Introduction

Sickle cell disease (SCD) is a monogenetic disease that is associated with several phenotypes[1] including sickle cell anemia (SCA) and is complicated by an increased predisposition to infection. SCA results from a mutation in the human β-globin gene leading to the substitution of valine for glutamic acid at the 6th position of the globin chain in the hemoglobin (Hb) molecule.[2] A consequence of this mutation is formation of Hb polymers, which cause the red blood cells (RBCs) to assume a ''sickle'' shape during periods of hypoxia, acidosis, excessive stress, or dehydration. Sickle RBCs block the microcirculation, either physically or by inducing vessel injury and thrombosis, resulting in end organ ischemia and damage.[3] Patients with SCA display features of under nutrition, including inadequate growth in height, reduced lean body mass, delayed pubertal development, and decreased serum levels of micro- and macronutrients.[4–7] These features occur despite the fact that there is no difference in caloric intake between individuals with SCA and normal healthy controls, suggesting that the mechanism of under nutrition in SCA might not be related to inadequate intake.[6,8,9] It has been reported that increased metabolic demand, possibly from increased; myocardial activity (a compensation for anemia), erythropoiesis and protein metabolism are among the factors responsible for a state of relative nutrient deficit in SCA.[10–12]

Under nutrition in general is associated with poor immune function and is consequently regarded as the most common cause of immunodeficiency worldwide.[13] The frequency and type of infections observed among patients with SCA are similar to those observed for

non-SCA patients with malnutrition,[14,15] further underscoring the earlier argument associating SCA with under nutrition. Non-SCA patients with malnutrition have decreased immune function and deficient acute inflammatory cytokines, similar to those reported for SCA patients.[16,17] Consequently, the observed deficiency in recruiting acute inflammatory proteins required for effective immune response to infections in individuals with SCA may be due in part, to under nutrition induced by the increased nutritional/caloric demands associated with SCA hypermetabolism. Additionally, the subclinical ischemic endothelial injury induced by sickle RBCs and hypoxia produce a chronic subclinical inflammatory response,[18] which may compromise the acute phase response to infection. In particular, studies show that serum from individuals with SCA displays decreased opsonic activity (the process of targeting antigens for phagocytosis/destruction by activated compliment factors), resulting in decreased compliment fixation.[19,20] Besides, patients with SCA show decreased ability to activate the alternate complement pathway[21] required for containing infection by capsular organisms. Both processes (opsonization and activation of the alternate complement pathway) require the activity of acute inflammatory cytokines such as tumor necrosis factor (TNF)-α and interferon (IFN)-γ.[16,22]

Zinc deficiency has also been well documented in individuals with SCA and, as in otherwise healthy zinc deficient controls,[23] results in decreased thymulin activity, low interleukin (IL)-2 level, low CD_4^+/CD_8^+ T-cell ratio, and deceased expression of IFN-γ and TNF-α.[24–26] Zinc supplementation resulted in improved peripheral levels of IFN-γ (required for major histocompatibility complex (MHC) class I antigen expression) and promotion of the initial acute inflammatory response needed for mounting a defense against microbial infection.[27] Zinc supplementation also resulted in decreased frequency of hospitalization and infection among individuals with SCD.[28,29]

Heyman et al.,[6] demonstrated that providing children with SCA and growth retardation with additional calories/protein supplements by nasogastric (NG) intubation or as oral nightly formulas for at least six weeks led to significant improvement in growth rates, reduced pain episodes, and decreased frequency of infection in the NG tube group; the group receiving oral supplement only had decreased episodes of pain and infection compared with SCA controls who showed no improvement in these clinical parameters after receiving only vitamin and mineral supplements. The mechanism by which additional calories could result in decreased frequency of infection in individuals with SCA is still not well documented.

More recently, our laboratory has demonstrated that sickle mice fed a high protein diet (35% of calories from protein) experienced improved weight gain compared with those maintained on a normal mouse diet supplying 20% of calories from protein.[30,31] Furthermore, malnourished mice have been shown to have decreased expression of Toll-like receptor 4 (TLR-4), which along with TNF-α mediates the acute inflammatory response to infection. In addition, malnourished mice show decreased expression of TNF-α compared with controls.[16,32] With this information in mind, we hypothesized that SCA mice fed with a high protein diet (35% calories from protein) would show increased plasma levels of T helper cell 1 (TH1) and consequently TH2 associated cytokines, compared with controls. We tested this hypothesis by using a longitudinal cross sectional study with transgenic SCA mice expressing exclusively human sickle Hb.[33]

Methods

Transgenic mice

Based on prior data, it was determined that a sample size of eight mice per group would be needed to achieve a power of 0.8, at an alpha of 0.05 with a 95% confidence interval. The Berkeley transgenic sickle cell mice (of mixed genetic background) had been determined by prior studies to very closely replicate human SCA and its complications[34] and was the model used in this study, with C57BL/6 mice as controls. A total of 32 weanling mice (16 C57BL/6 and 16 transgenic sickle cell−SCA, ~4 weeks old) were randomly allocated to one of four groups, to receive either the 20% protein standard mouse diet or a 35% protein enriched diet (Purina Feeds LLC, St. Louis, MO). These are iso-caloric diets providing either 20% or 35% of calories as protein by adjusting the dextrin concentration.[30] Hence, eight C57BL/6 or control mice were randomized to the 20% protein diet and were labeled C20 and 8–35% protein (C35). Similarly, SCA mice were randomized to either the 20% (S20, $n=8$) or 35% protein diet (S35, $n=8$). All mice were fed for three months following one week of adaptation to the diet and housing environment. A re-designed metabolic cage, which allows for use of bedding, required for preventing exposure of the mice to hypothermia and more accurate measurement of the feed consumed than conventional metabolic cages was used for this experiment. The cage design permitted collection and subtraction of the gnaw waste from the total feed weight provided to the mice in each cage. All procedures were approved by the Institutional Animal Care and Research Committees of Emory University and Morehouse School of Medicine, which reviewed the protocol.

Procedure

Daily food intake per cage was used to approximate the average daily food intake per mouse per week in the same cage. Concurrent weekly individual body weights were utilized to compute rates of weight gain[30] over the three-month feeding period. The total weight gained was then divided by the total time of feeding and the total feed consumed to yield the weight gained per gram of feed consumed per day, or rate of weight gain (ROWG). Near the end of the study period (usually 3 days prior), blood was taken either via the central tail vein or by retro-orbital sampling, for complete blood count (CBC) using Hema True® veterinary hematology analyzer (Heska Inc., Loveland, CO) and reticulocyte count/percent using flow cytometry. The mice were sacrificed for specimen collection by isoflurane anesthesia and cervical dislocation. Blood samples were collected via cardiac puncture into sodium EDTA tubes and the plasma was

immediately separated by centrifugation at 4°C. The plasma was divided into 100 μL aliquots and stored at −80°C until analyzed for TH1 (IFN-γ, TNF-α, IL-1β, IL-6, and IL-13) and TH2 (IL-4 and IL-10) associated cytokines, which were paneled and assayed alongside chemokine IP10/CXCL10 and growth factors granulocyte-macrophage colony-stimulating factor (GMCSF) and vascular endothelial growth factor (VEGF), using multiplex antibody immobilized beads (Millipore Corp, Billerica, MA). The fluorescent intensity and concentration of the cytokines were determined by a Bioplex system (Bio-Rad, Hercules, CA), using 5PL interpolated logistic curve generated using manufacturer supplied standards. Food intake per mouse was used to standardize the plasma values for the cytokines.

Data analysis

Data analysis was carried out using GraphPad Prism v5 and SPSS v20 for Windows®. The differences in mean ROWG, hematological parameters, and plasma cytokine levels between groups were evaluated using ANOVA. The cytokine levels were standardized using the amount of feed consumed to adjust for variation in cytokine level attributable to difference in amount of feed consumed. Pearson correlation was used to test for association between plasma cytokine level and ROWG. Results were expressed in tables as means ± SD in tables, with a P value < 0.05 considered statistically significant.

Results

Weight gain

On average, S35 had improved weight gain per gram of feed consumed per day (ROWG) compared with S20, C35, and C20 but this result was not statistically significant, $P > 0.05$ (Table 1). The S35 showed an average of 43.9% improvement in ROWG over the period of feeding, compared with S20. As expected the weight gain for the C35 group was less than for the C20 group, because the high protein diet is metabolically toxic or burdensome for control mice,[30] but this difference was not statisitically signficant.

Hematology

As expected, the C20 and C35 mice had significantly higher RBC count and Hb levels compared with the S20 and S35 (Table 1). Conversely, both white blood cell (WBC) count and reticulocyte percentage were significantly higher for S mice than C mice ($P < 0.05$). The S35 had a slightly higher mean RBC count than the S20 ($P = 0.09$) and a slightly higher mean Hb level compared with S20 ($P = 0.09$). Also, S35 had a slightly higher mean WBC count ($P = 0.08$) but a slightly lower mean reticulocyte percent ($P = 0.08$) than the S20 (Table 1).

Plasma cytokines

In general, S35 had significantly higher levels of both TH1 and TH2 linked cytokines and chemokine compared with S20. As shown in Table 2, plasma TNF-α level was significantly higher for S35 compared with S20 ($P = 0.02$), C35 ($P = 0.04$), and C20 ($P < 0.01$). Similarly, plasma IFN-γ level was significantly higher among S35 compared with S20 ($P = 0.01$) and C20 ($P < 0.01$). Following the same pattern, the average plasma level for IL-10 was significantly higher among the S35 compared with S20 ($P = 0.02$), C20 ($P < 0.01$), and C35 ($P < 0.01$). The mean plasma IL-4 level was also significantly higher among the S35 compared with S20 ($P = 0.01$), as was the mean plasma GMCSF level ($P = 0.03$).

Correlations

Significant negative correlation was obtained for the C35 mice between ROWG and serum IL-6 levels ($r = -0.77$, $P = 0.04$), whereas IFN-γ levels tended to be negatively correlated with ROWG ($r = -0.74$, $P = 0.05$). In contrast for the S20 mice, ROWG tended to be positively correlated with plasma levels of IFN-γ ($r = 0.67$, $P = 0.08$), IL-4 ($r = 0.68$, $P = 0.06$) and VEGF ($r = 0.66$, $P = 0.09$). However, significant positive correlation was obtained in S35 mice between ROWG and serum VEGF levels ($r = 0.82$, $P = 0.03$).

Discussion

It is well known that laboratory mice have optimal growth when fed 20% of energy from protein and standard rodent chow has approximately this composition.[35] However, in a previous study, we demonstrated that S mice, which tend to be smaller and have a slower ROWG on standard rodent chow than age matched controls, required more calories from protein and gained weight faster than the controls when fed 35% of energy from protein,[30] see Table 1. Additionally, in this study, we demonstrated that the high

Table 1 Average rate of weight, gain, i.e. change in weight per gram of feed consumed per day for mice fed a particular diet and changes in hematological parameters[a]

Group	ROWG ($\mu g \cdot g\ feed^{-1} \cdot d^{-1}$)	RBC count ($10^6/\mu L$)	Hemoglobin (g/dL)	WBC count ($10^3/\mu L$)	Reticulocytes (%)
C20	491.3 ± 77.2	10.4 ± 1.4	15.5 ± 1.7	8.6 ± 2.3	2.6 ± 0.3
C35	457.5 ± 258.2	11.1 ± 0.5	16.9 ± 1.5	8.3 ± 1.2	4.2 ± 0.4[b]
S20	368.8 ± 149.6	6.8 ± 0.77[b]	9.98 ± 0.49[b,c]	16.12 ± 1.48[b,c]	39.16 ± 7.13[b,c]
S35	531.3 ± 242.7	7.7 ± 0.59[b]	10.56 ± 1.09[b,c]	22.24 ± 7.00[b,c]	34.74 ± 8.80[b,c]

C20: controls on 20% protein energy diet; C35: controls on 35% protein energy diet; S20: sickle cell mice on 20% protein energy diet; S35: sickle cell mice on 20% protein energy diet.

[a]Mice were fed for three months. Values are mean ± SD, $n = 8$.

[b]Indicates $P < 0.05$ for comparison with C20 mice.

[c]Indicates $P < 0.05$ for comparison C35 mice.

Table 2 Comparison of plasma level of cytokines per gram of feed consumed per day by type of feed and mouse phenotype[a]

Group	TNF-α ($\times 10^{-4}$ pg/mL/g feed^{-1})	IFN-γ ($\times 10^{-4}$ pg/mL/g feed^{-1})	IL-10 ($\times 10^{-4}$ pg/mL/g feed^{-1})	IL-4 ($\times 10^{-4}$ pg/mL/g feed^{-1})	GMCSF ($\times 10^{-4}$ pg/mL/g feed^{-1})
C20	243.1 ± 200.4	77.5 ± 75.9	74.3 ± 147.6	76.6 ± 101.5	74.3 ± 147.6
C35	336.4 ± 193.1	194.7 ± 231.4	692.5 ± 809.4[b]	221.3 ± 326.0	692.5 ± 809.0
S20	299.0 ± 258.6	134.7 ± 127.2	1635.1 ± 1066.2[b]	53.0 ± 51.6	592.1 ± 667.0
S35	661.8 ± 273.0[b,c,d]	300.1 ± 60.5[b,d]	3201.9 ± 1302.0[b,c,d]	110.3 ± 10.6[d]	1322.2 ± 503.5[d]

C20: controls on 20% protein energy diet; C35: controls on 35% protein energy diet; S20: sickle cell mice on 20% protein energy diet; S35: sickle cell mice on 20% protein energy diet.

[a]Mice were fed for three months and cytokine levels are expressed per gram of feed consumed to account for variations in cytokine levels that might be due to variations in the amount of feed consumed. Values are mean ± SD, $n = 8$.

[b]Indicates $P < 0.05$ for comparison with C20 mice.

[c]Indicates $P < 0.05$ for comparison C35 mice.

[d]Indicates $P < 0.05$ for comparison with S20 mice.

protein/energy diet (35%) was associated with increased plasma levels of markers of acute inflammatory response, and may be optimal for SCA mice. Our current study provides insight into a possible mechanism by which the comprehensive approach to dietary supplementation by Heyman et al.,[6] resulted in decreased incidence of infection among individuals with SCA, in that the additional nutrient supply may have provided the needed substrate for mounting a sufficient innate immune response to provide immediate defense against infection. Although not statistically significant, our findings concur with the previous report of improvement in weight gain[30] in transgenic sickle mice maintained on a 35% protein diet.

Most therapy for the management of SCA and preventing complications are targeted towards improvement in the hematological profile (Hb level) of the patients, which has been associated with decreased risk of SCA complications such as stroke and frequent infection.[36] Our data in Table 1 show that there was an improvement in the RBC count for S35 compared with S20 ($P = 0.09$). Although this improvement was not statistically significant, it shows a trend, indicating that the 35% test diet may be associated with prolongation of RBC survival. Additionally, in the management of SCA, the aim is for a modest improvement in hematological parameters like RBC count or Hb level because of the negative consequences (e.g. stroke and heart failure) of a drastic increase in these parameters. There was also a slightly decreased reticulocyte percentage for S35 compared with S20 (Table 1), which is expected, supporting the slight increase in RBC count, and implies a decrease in hemolysis in S35 compared with S20 and consequent reduced rate of erythropoiesis and reticulocyte percent as demonstrated here (Table 1). Increased reticulocyte percentage is a response to increased hemolysis, which is a cardinal feature of SCA. There was also a slight increase in Hb level for S35 compared with S20, further strengthening the observation of a higher RBC count for S35 compared with S20. As expected, the S mice had higher WBC count compared with C mice. But additionally, the S35 had a slightly higher WBC count than the S20 ($P = 0.08$). This difference may be related to improved proliferation in response to increased cytokine levels (IFN-γ is associated with proliferation and maturation of WBCs). Although individuals with SCA have a higher baseline WBC count, there is evidence suggesting that the cells are not fully functional.[37] More research is needed to confirm the definite mechanism of the slight increase in the WBC count among S35 over S20, and also their level of functionality in immune response.

Prior studies have suggested a defective acute inflammatory response to infection among individuals with SCA.[38–41] Furthermore, it is well established that children with SCA are in a state of under nutrition not due to inadequate dietary intake, but due to increased metabolic demand for nutrients.[7] The results of this present study show increased plasma levels of TNF-α and IFN-γ, both cytokines are involved in the acute inflammatory response required for resisting and eliminating infection.[42–44] Wild type mice maintained on a low protein diet showed decreased expression of TLR-4 and lymphocyte antigen 96 also known as MD-2,[16] both of which along with CD-14 were found to be involved with stimulating the expression of TNF-α,[42–44] These malnourished mice also had a decreased expression of TNF-α and other inflammatory cytokines involved in the acute inflammatory reaction to infection or infectious material.[16] In this present study, plasma IFN-γ level was significantly higher for S35 mice compared with the other groups. Messenger ribonucleic acid (mRNA) expression of this cytokine is reportedly low among under nourished individuals and those with protein energy malnutrition.[17] The low steady state plasma level of this cytokine is still debatable, given the prevalence of zinc deficiency among individuals with SCA,[25] and the relationship between zinc and the expression of this cytokine. However, some studies have documented elevated plasma levels of IFN-γ at steady state, but not during crisis.[45] IFN-γ plays a key role in the regulation of immune cells and also increased expression of TNF-α.[22,46] Despite the potential immunologic benefit of elevated TNF-α, it has been demonstrated to be associated with endothelial activation in the context of atherosclerosis.[47] This activity, if it occurs in SCD patients could potentially precipitate or potentiate a vaso-occlusive episode. In addition, it stimulates increased expression of IL-4.[22] Our data have revealed both elevated IL-4 and IFN-γ levels for the S35 mice, which concurs with previous findings and the reported relationship between these cytokines. Further studies are needed to elucidate the implication(s) of this

finding. IL-10 levels are usually elevated among individuals with SCA compared with non-SCA,[48] and our finding of higher IL-10 levels in the SCA mice compared with controls is in accord. In addition, S35 had significantly elevated IL-10 levels compared with all the other groups (Table 2). IL-10 is known to play an anti-inflammatory role in certain situations[49,50] and we reasoned that its elevation in the S35 compared with S20 mice might be a response to the elevated levels of TNF-α and IFN-γ, made possible by additional substrate availability for protein synthesis via more calories from protein in the diet.

The negative correlation between IL-6 and ROWG in the C35 mice is not entirely surprising – as these animals continue only limited weight gain during the excessive protein intake,[51] Table 1. High levels of IL-6, a pro-inflammatory cytokine, are generally associated with muscle wasting, and might in part explain the observed negative correlation with ROWG.[42] The significant correlation between ROWG and plasma VEGF level among S35, with an additional significantly higher level of GMCSF among S35 compared with S20 might be a reflection of the higher RBC/lower reticulocyte count and higher WBC count respectively. Both molecules are involved in the maintenance of angiogenesis, improvement in tissue oxygen supply via the NO pathway (VEGF) and improvement in immunity via leukocyte proliferation (GMCSF).[52–54] In a separate study, we have shown that 35% diet resulted in significant improvement in lean body mass (submitted). This might explain in part, the correlation observed among the S35, since VEGF is also associated with improvement in muscle oxygen supply via new vessel formation.[52] Further studies are needed to confirm the findings of this study regarding improvement in immunologic activity due to micro- and macronutrient dietary supplementation in SCA.

Conclusion

A high protein diet gleaned modest improvements in weight gain, RBC count, Hb level, and a decreased reticulocyte percent in Berkeley SCA mouse model. Furthermore, the high protein diet was associated with a significantly higher plasma level of TH1 (TNF-α and IFN-γ) and TH2 (IL-4 and IL-10) associated cytokines, essential for containing and eliminating infections. Based on these results, we concluded that decreased infectious episodes reported for individuals with SCA receiving additional protein/calorie supplementation, may have resulted from increased expression of protein mediators (cytokines, chemokines, and growth factors) of the acute inflammatory response to infection, because of additional substrate availability derived from the supplement, for synthesis of these acute phase proteins.

Author contributions: This work has been done with significant contributions from those listed here as authors. JMH conceived and designed the study, DRA provided mice for the experiments and HIH and PLC conducted the experiment. JMH and DRA provided supervision. HIH and JMH wrote the manuscript, but all authors approved the final version of the manuscript for submission.

DECLARATION OF CONFLICTING INTEREST

The metabolic cage used for this experiment was conceived and designed in JMH's laboratory and is patent protected. The other authors have no relevant conflict of interest to declare.

ACKNOWLEDGEMENTS

This project was funded by grant from the National Institute of Health (NIH/NHLBI R21HL092358 and NIH/NCRR 5P20RR0111044 pilot to JMH). Also in part by MUSC/ Department of Defense Cooperative Agreement (SE VIEW) number: W81XWH-10-2-0057.

REFERENCES

1. Driss A, Asare KO, Hibbert JM, Gee BE, Adamkiewicz TV, Stiles JK. Sickle cell disease in the post genomic era: a monogenic disease with a polygenic phenotype. *Genomics Insights* 2009;**2**:23–48
2. Pauling L, Itano HA. Sickle cell anemia a molecular disease. *Science* 1949;**110**:543–8
3. Aster JC. Disease of organs systems: red blood cells and bleeding disorders – sickle cell disease. In: Vinay K, Fausto N, Abbas AK (eds). *Robins and Cotran Pathologic Basis of Disease*, 7 ed. Philadelphia: Elselvier Saunders, 2005:628–32
4. Buison AM, Kawchak DA, Schall J, Ohene-Frempong K, Stallings VA, Zemel BS. Low vitamin D status in children with sickle cell disease. *J Pediatr* 2004;**145**:622–7
5. Henderson RA, Saavedra JM, Dover GJ. Prevalence of impaired growth in children with homozygous sickle cell anemia. *Am J Med Sci* 1994;**307**:405–7
6. Heyman M, Katz R, Hurst D, Chiu D, Ammann A, Vichinsky E, Gaffield B, Castillo R, Kleman K, Thaler MM, Lubin B. Growth retardation in sickle cell disease treated by nutritional support. *Lancet* 1985;**325**:903–6
7. Hyacinth HI, Gee BE, Hibbert JM. The role of nutrition in sickle cell disease. *Nutr Metab Insights* 2010;**3**:57–67
8. Gray NT, Bartlett JM, Kolasa KM, Marcuard SP, Holbrook CT, Horner RD. Nutritional status and dietary intake of children with sickle cell anemia. *J Pediatr Hematol/Oncol* 1992;**14**:57–61
9. Zemel BS, Kawchak DA, Ohene-Frempong KWAK, Schall JI, Stallings VA. Effects of delayed pubertal development, nutritional status, and disease severity on longitudinal patterns of growth failure in children with sickle cell disease. *Pediatr Res* 2007;**61**:607–13
10. Hibbert JM, Creary MS, Gee BE, Buchanan I, Quarshie A, Hsu LL. Erythropoiesis and myocardial energy requirements contribute to the hypermetabolism of childhood sickle cell anemia. *J Pediatr Gastroenterol Nutr* 2006;**43**:680–7
11. Hibbert JM, Hsu LL, Bhathena SJ, Irune I, Sarfo B, Creary MS, Gee BE, Mohamed AI, Buchanan ID, Al-Mahmoud A, Stiles JK. Proinflammatory cytokines and the hypermetabolism of children with sickle cell disease. *Exp Biol Med* 2005;**230**:68–74
12. Salman EK, Haymond MW, Bayne EDWA, Sager BK, Wiisanen ADA, Pitel PAUL, Darmoun DOMI. Protein and energy metabolism in prepubertal children with sickle cell anemia. *Pediatr Res* 1996;**40**(1):34–40
13. Katona P, Katona-Apte J. The interaction between nutrition and infection. *Clin Infect Dis* 2008;**46**:1582–8
14. Booth C, Inusa B, Obaro SK. Infection in sickle cell disease: a review. *Int J Infect Dis* 2010;**14**:e2–12
15. Lesourd BM, Mazari L. Immune responses during recovery from protein-energy malnutrition. *Clin Nutr* 1997;**16**:37–46
16. Fock RA, Vinolo MAR, de Moura Sá Rocha V, de Sá Rocha LC, Borelli P. Protein-energy malnutrition decreases the expression of TLR-4/MD-2

and CD14 receptors in peritoneal macrophages and reduces the synthesis of TNF-α in response to lipopolysaccharide (LPS) in mice. *Cytokine* 2007;**40**:105–14
17. González-Martínez H, Rodríguez L, Nájera O, Cruz D, Miliar A, Domínguez A, Sánchez F, Graniel J, González-Torres MC. Expression of cytokine mrna in lymphocytes of malnourished children. *J Clin Immunol* 2008;**28**:593–9
18. Hebbel RP, Osarogiagbon R, Kaul D. The endothelial biology of sickle cell disease: inflammation and a chronic vasculopathy. *Microcirculation* 2004;**11**:129–51
19. Bjornson AB, Gaston MH, Zellner CL. Decreased opsonization for Streptococcus pneumoniae in sickle cell disease: studies on selected complement components and immunoglobulins. *J Pediatr* 1977;**91**:371–8
20. Winkelstein JA, Drachman RH. Deficiency of pneumococcal serum opsonizing activity in sickle-cell disease. *N Engl J Med* 1968;**279**:459–66
21. Johnston RB, Newman SL, Struth AG. Increased susceptibility to infection in sickle cell disease: defects of opsonization and of splenic function. *Birth Defects Orig Artic Ser* 1975;**11**:322–7
22. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-γ: an overview of signals, mechanisms and functions. *J Leukoc Biol* 2004;**75**:163–89
23. Prasad AS, Meftah S, Abdallah J, Kaplan J, Brewer GJ, Bach JF, Dardenne M. Serum thymulin in human zinc deficiency. *J Clin Invest* 1988;**82**:1202–10
24. Prasad AS. *Acquired Zinc Deficiency and Immune Dysfunction in Sickle Cell Anemia*. New York: Marcel Dekker, 1993
25. Prasad AS. Zinc deficiency in patients with sickle cell disease. *Am J Clin Nutr* 2002;**75**:181–2
26. Prasad AS, Kaplan J, Brewer GJ, Dardenne M. Immunological effects of zinc deficiency in sickle cell anemia (SCA). *Prog Clin Biol Res* 1989;**319**:629–47. (discussion 48–9)
27. Prasad AS. Zinc and immunity. *Mol Cell Biochem* 1998;**188**:63–9
28. Bao B, Prasad AS, Beck FWJ, Snell D, Suneja A, Sarkar FH, Doshi N, Fitzgerald JT, Swerdlow P. Zinc supplementation decreases oxidative stress, incidence of infection, and generation of inflammatory cytokines in sickle cell disease patients. *Transl Res* 2008;**152**:67–80.
29. Prasad AS, Beck FWJ, Kaplan J, Chandrasekar PH, Ortega J, Fitzgerald JT, Swerdlow P. Effect of zinc supplementation on incidence of infections and hospital admissions in sickle cell disease (SCD). *Am J Hematol* 1999;**61**:194–202
30. Archer DR, Stiles JK, Newman GW, Quarshie A, Hsu LL, Sayavongsa P, Perry J, Jackson EM, Hibbert JM. C-reactive protein and interleukin-6 are decreased in transgenic sickle cell mice fed a high protein diet. *J Nutr* 2008;**138**:1148–52
31. Capers PL, Hyacinth IH, Cue S, Chappa P, Archer DR, Hibbert JM. Effect of high protein diet on transgenic sickle mice. *FASEB J* 2010;**24**:lb394
32. Stenvinkel P, Ketteler M, Johnson RJ, Lindholm B, Pecoits-Filho R, Riella M, Heimburger O, Cederholm T, Girndt M. IL-10, IL-6, and TNF-α: central factors in the altered cytokine network of uremia[mdash]The good, the bad, and the ugly. *Kidney Int* 2005;**67**:1216–33
33. Pászty C, Brion CM, Manci E, Witkowska HE, Stevens ME, Mohandas N, Rubin EM. Transgenic knockout mice with exclusively human sickle hemoglobin and sickle cell disease. *Science* 1997;**278**:876–8
34. Manci EA, Hillery CA, Bodian CA, Zhang ZG, Lutty GA, Coller BS. Pathology of Berkeley sickle cell mice: similarities and differences with human sickle cell disease. *Blood* 2006;**107**:1651–8
35. Reeves PG, Nielsen FH, Fahey GC Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr* 1993;**123**:1939–51
36. Adams RJ, McKie VC, Hsu L, Files B, Vichinsky E, Pegelow C The STOP Investigators. Prevention of a first stroke by transfusions in children with sickle cell anemia and abnormal results on transcranial doppler ultrasonography. *N Engl J Med* 1998;**339**:5–11
37. Qari MH, Zaki WA. Flow cytometric assessment of leukocyte function in sickle cell anemia. *Hemoglobin* 2011;**35**:367–81
38. Obaro S. Pneumococcal infections and sickle cell disease in Africa: does absence of evidence imply evidence of absence? *Arch Dis Child* 2009;**94**:713–6
39. Onwubalili JK. Sickle cell disease and infection. *J Infect* 1983;**7**:2–20
40. Tamouza R, Neonato M-G, Busson M, Marzais F, Girot R, Labie D, Elion J, Charron D. Infectious complications in sickle cell disease are influenced by HLA class II alleles. *Hum Immunol* 2002;**63**:194–9
41. Thorell EA, Sharma M, Jackson MA, Selvarangan R, Woods GM. Disseminated nontuberculous mycobacterial infections in sickle cell anemia patients. *J Pediatr Hematol/Oncol* 2006;**28**:678–81
42. Fujita J, Tsujinaka T, Ebisui C, Yano M, Shiozaki H, Katsume A, Ohsugi Y, Monden M. Role of interleukin-6 in skeletal muscle protein breakdown and cathepsin activity in vivo. *Eur Surg Res* 2008;**28**:361–6
43. Miyake K. Innate recognition of lipopolysaccharide by CD14 and toll-like receptor 4-MD-2: unique roles for MD-2. *Int Immunopharmacol* 2003;**3**:119
44. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 1998;**282**:2085–8
45. Pathare A, Al Kindi S, Alnaqdy AA, Daar S, Knox-Macaulay H, Dennison D. Cytokine profile of sickle cell disease in Oman. *Am J Hematol* 2004;**77**:323–8
46. Luster AD, Unkeless JC, Ravetch JV. γ-Interferon transcriptionally regulates an early-response gene containing homology to platelet proteins. *Nature* 1985;**315**:672–6
47. McKellar GE, McCarey DW, Sattar N, McInnes IB. Role for TNF in atherosclerosis? Lessons from autoimmune disease. *Nat Rev Cardiol* 2009;**6**:410–7
48. Lanaro C, Franco-Penteado CF, Albuqueque DM, Saad ST, Conran N, Costa FF. Altered levels of cytokines and inflammatory mediators in plasma and leukocytes of sickle cell anemia patients and effects of hydroxyurea therapy. *J Leukoc Biol* 2009;**85**:235–42
49. Mocellin S, Panelli MC, Wang E, Nagorsen D, Marincola FM. The dual role of IL-10. *Trends Immunol* 2003;**B**:36–43
50. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 2001;**19**:683–765
51. Jackson A. Limits of adaptation to high dietary protein intakes. *Eur J Clin Nutr* 1999;**53**:S44–52
52. Afuwape AO, Kiriakidis S, Paleolog EM. The role of the angiogenic molecule VEGF in the pathogenesis of rheumatoid arthritis. *Histol Histopathol* 2002;**17**:961–72
53. Patan S. Vasculogenesis and angiogenesis. In: Kirsch M, Black P (eds). *Angiogenesis in Brain Tumors*. USA: Springer, 2004:3–32
54. Hamilton JA, Anderson GP. GM-CSF biology. *Growth factors (Chur, Switzerland)* 2004;**22**:225–31

(Received May 3, 2013, Accepted September 3, 2013)