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C-Reactive Protein and Interleukin-6 Are Decreased in Transgenic Sickle Cell Mice Fed a High Protein Diet[1,,2]

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Abstract

Sickle cell disease is associated with hypermetabolism and a consequent shortage of substrates for normal growth and healthy immune response. The protein:energy ratio is a major determinant of dietary adequacy; the requirement for optimal growth of control mice is 20% of energy from dietary protein. This study investigated the efficacy of increased dietary protein for improving weight gain and reducing inflammation in the Berkeley sickle cell mouse model (S). The study examined the effect of diet on weight gain and circulating levels of 2 inflammatory proteins, C-reactive protein (CRP), and cytokine interleukin-6 (IL-6). Male C57BL/6 (C) control ($n = 8$) and S mice ($n = 8$) were randomized at weaning to 40 d of isoenergetic diets containing 20% (normal) and 35% (high) of energy from protein (C20, C35, S20, S35), replacing dextrin. Rate of weight gain was calculated and plasma CRP and IL-6 concentrations determined by ELISA. Liver mRNA expression of these proteins was measured by real-time PCR and L-arginase by colorimetric assay. S35 mice tended to gain weight more rapidly than S20 mice ($P = 0.06$) and more rapidly than C35 mice ($P < 0.01$). Circulating CRP and IL-6 levels were also lower in S35 mice than in S20 mice ($P < 0.05$), as was liver CRP mRNA expression ($P < 0.01$). These results demonstrate that introducing a high protein diet at weaning attenuates the steady-state inflammation in this S mouse model. Dietary L-arginine availability was investigated as a possible mechanism for increased nitric oxide production and consequent reduced inflammation.

Introduction

Homozygous sickle cell disease (HbSS)[6] is characterized by chronic hemolysis and anemia despite a compensatory increase of erythropoiesis. In the natural history of this disease, the anemia does not resolve; sickle red cells (RBC) have a very short half-life and lyse or are

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[6]Abbreviations used: C, C57BL/6 control mice; C20, control mice fed 20% protein diet; C35, control mice fed 35% protein diet; CRP, C-reactive protein; HbAA, normal hemoglobin genotype; HbSS, homozygous sickle cell disease; IL-6, interleukin-6; S, sickle mice; S20, sickle mice fed 20% protein diet; S35, sickle mice fed 35% protein diet.

removed from the circulation. The increased protein synthesis and catabolism (1–6) directed toward red cell replacement constitutes a persistent drain on protein/ energy substrates that is likely to cause nutrient shortage, particularly of amino acids vital for normal growth and development (7–10). The chronic hemolysis also induces inflammation, even among steady-state HbSS patients (2,11,12), presumably through subclinical vascular endothelial injury and transient vasoocclusive events (13). Basal leukocytosis is typical and is an important independent risk factor for disease severity (14). Acute phase reactants such as C-reactive protein (CRP) are elevated among patients at steady state (2,11,12) as well as during painful vasoocclusive crisis (15). Using stepwise regression analysis, we recently demonstrated that inflammation, via CRP, could predict increased energy expenditure in HbSS children (2). Elevated protein breakdown is reported in HbSS (1,4–6,16) and the process of releasing amino acids required for the synthesis of acute phase proteins and cytokines may be driven by proinflammatory cytokines such as interleukin-6 (IL-6) (17).

Reports in the literature demonstrate that individuals with HbSS are hypermetabolic, with elevated protein turnover and energy expenditure in affected children (1,2,16), adolescents (3), and adults (4) compared with healthy individuals having the normal hemoglobin genotype (HbAA). In addition, we have demonstrated increased urea production in adults with HbSS (5,6), suggesting increased net protein catabolism. Growth and development are suboptimal (10) in HbSS and body mass is reduced (2–4) compared with HbAA controls, yet dietary intake is not usually increased in HbSS (4,8). These observations suggest an increased dietary requirement to satisfy increased metabolic needs in HbSS (7).

Although failure of adequate childhood growth and development (18,19) and abnormally low body mass (2–4) are well-known symptoms of HbSS, much of the research on nutritional deficiencies in this disease has been focused on vitamin and mineral micronutrients associated with maintenance of erythropoietic and redox potentials of the RBC (20). This focus has persisted despite evidence from a small prospective study of growth-retarded children (21), that daily vitamin and mineral supplements alone did not improve the clinical status or growth in childhood sickle cell disease. In contrast, children receiving a balanced food supplement orally showed improved clinical status. Furthermore, nasogastric administration of the supplement was associated with rapid weight gain in addition to the clinical improvement. Because it is generally considered that increased hemolytic rate and consequent elevated erythropoiesis are central to the development of increased protein and energy requirement in HbSS (1–8,18), it is our hypothesis that increasing these macronutrients could help to decrease protein catabolism, promote normal growth and development, and correct other metabolic abnormalities, such as baseline inflammation in children with the disease. We tested this hypothesis in the Berkeley sickle cell mouse model (S) by investigating whether increasing the percentage of energy from dietary protein could improve weight gain and lower inflammation in weanling mice.

Animals and Methods

Mice

Although several mouse models have been developed to mimic human sickle cell disease, the Berkeley model developed by Paszty et al. (22) has the most severe hemolytic anemia, sickle deformation of erythrocytes, chronic inflammation, oxidative stress, pulmonary hypertension, and priapism (22–29). The Berkeley mouse model expresses exclusively human sickle hemoglobin because of targeted deletions of murine α and β globins with a transgene containing human α, β^S, $^A\gamma$, and $^G\gamma$ globins (22,30), on a mixed genetic background (FVB/N, 129, DBA/2, C57BL/6, Black Swiss). The S mice used for these experiments were obtained from a colony established at Emory University School of Medicine, with founders from Lawrence Berkeley National Laboratory, Berkeley, CA,

kindly provided by Drs. Narla Mohandas and Edward Rubin. Male S and control C57BL/6 (C) mice were housed (4 per cage) in cages specially designed in our laboratory to permit effective separation of food from urine and feces, plus use of bedding to prevent increased mortality of S mice by exposure to hypothermia. The configuration enabled more precise measurement of food consumption than a conventional metabolic cage. The S mice were maintained in a pathogen-free environment. All procedures were approved by the Institutional Animal Care and Use Committees of Emory University and Morehouse School of Medicine, which approved the protocols.

Study design

The study was designed as a cross-sectional controlled feeding trial. It is well known that laboratory mice have optimal growth when fed 20% of energy from protein and standard rodent chows have approximately this composition (31). An initial experiment feeding the mice a series of diets ranging from 15 to 35% of energy from protein showed that 35% protein was optimal for weight gain in the S mice. Therefore, 20% (normal) and 35% (high) protein diets were studied further[7]. Two studies of 4 mice per group were conducted. The number of mice varied from 3 to 8, because some had erroneous hemoglobin phenotypes or gender and so were excluded. The arginine availability study included 3–7 S mice per group. All mice (4 wk old) were weaned to the test diets (Purina Mills, Test Diet Division), which were fed as pellets to reduce spillage. After 2 wk of acclimation (6 wk of age), weekly total food intake corrected for spillage was measured, quantity of daily food intake per mouse was calculated, concurrent weekly individual body weights were recorded, and rate of weight gain was calculated. At the end of the study period (~40 d), the mice were killed for specimen collection using isoflurane and cervical dislocation, a rapid and virtually painless method of killing. Blood specimens were collected by cardiac puncture into prechilled tubes containing Na_2EDTA and the plasma was immediately separated by centrifugation at 4°C and 2000 × *g* for 10 min. The livers were harvested and stored in RNA Later solution (Ambion) to preserve RNA. All samples were stored frozen at −80°C until analysis. Plasma measurements were CRP, IL-6, and L-arginine and in the liver, mRNA expression of CRP and IL-6 and arginase activity.

IL-6, CRP protein, and mRNA measurements

The plasma concentration for IL-6 (detection limit 3 ng/L) and CRP were measured by ELISA using standard commercially available kits for mice (Biosource International and ALPCO Diagnostics, respectively). The tissues were thawed for mRNA quantification, weighed, then ground with molecular grade resin (ISC BioExpress) and Tri-Reagent (Molecular Research Center). Total RNA was isolated and the quantity and purity were determined. A 2-step RT-PCR was performed on the samples using primers for IL-6 and CRP normalized to 18S rRNA and measured by real-time PCR with the QuantiTect SYBR green PCR kit (Qiagen).

Circulating arginine and liver arginase activity

Plasma arginine levels were measured by HPLC (Hewlett Packard 1100, Agilent Technologies) as previously described (32), with the following modifications. We used a mobile phase consisting of 95:5 (v:v) acetonitrile-potassium phosphate buffer (pH 6.0). Samples (20 μL) were derivatized online at pH 8.5 and eluted from the column (Luna C18-

[7]Supplied by Purina Mills TestDiet Division (Richmond, IN). Twenty percent protein, TD 1812695 (g/kg diet): casein-vitamin free, 229.0; dextrin, 357.0; sucrose, 274.0; corn oil, 40.0; L-arginine, 8.0; energy (kJ/kg diet), 15648.0. Thirty-five percent protein, TD 1810032 (g/kg diet): casein-vitamin free, 386.0; dextrin, 89.0; sucrose, 360.0; corn oil, 65.0; L-arginine, 13.5; energy (kJ/kg diet), 16067.0. Identical components (g/kg diet): powdered cellulose, 50.0; mineral mix AIN-76 (31), 35.0; vitamin mix AIN-76A (31), 10.0; L-cysteine, 3.0; choline bitartrate, 2.0.

ODS, Phenomex) at a flow rate of 1.4 mL/min. Liver arginase activity was measured by conversion of L-arginine to urea at 37°C, pH 9.5, using an arginase colorimetric assay kit (BioAssay Systems). The liver (mean weight, 0.05 g) was homogenized in PBS buffer, centrifuged, and the supernatant diluted by 1:3000 prior to assay.

Rate of weight gain

Weight gain, g·g feed^{-1}·d^{-1}, was calculated over the period of most rapid growth just prior to the plateau in body weight, typically 40 d. This calculation allows for correction for variability from the possible adjustment of food intake by type of diet and for measurement during a period of rapid growth when the demand for nutrients is maximal.

Statistical analysis

A statistical test for normality using the sktest in STATA 9.2 was applied to continuous variables. These variables were not normally distributed ($P < 0.001$). Summary statistics for continuous variables are therefore presented as geometric means and 95% CI or means ± SD where indicated. Intergroup comparisons of continuous variables were made using the 2-sample Mann-Whitney-Wilcoxon rank sum test. $P < 0.05$ was considered significant for statistical testing, which was 2-tailed. Differences in rate of weight gain for S and C mice by diet type were assumed a priori based on existing literature (33); however, a statistical interaction test for proof of concept was performed by fitting a 2-way ANOVA model of the log-transformed weight gain outcome variable on mouse type, diet type, and mouse type × diet type (the interaction term). The resulting *P*-values for this model were assessed from the F-tests and the nominal value for significance of the interaction term was set at 0.05. The data were analyzed using STATA 9.2 (StataCorp) for Windows data analysis package.

Results

Weight change and rate of gain

Data from the preliminary experiments in which the mice were fed 15, 20, 25, and 35% of energy from protein (Table 1) showed C mice with most rapid weight gain at 20% protein, followed by a gradual decrease with increasing dietary protein. In contrast, the S mice gradually increased the rate of weight gain with increasing dietary protein. When only mice fed 20% and 35% protein diets were compared, the rate of weight gain was higher for the C mice fed 20% (C20) compared with 35% (C35) protein ($P < 0.01$). Conversely, S mice fed 35% protein (S35) tended to gain weight more rapidly than those fed 20% (S20) protein ($P = 0.064$) and more rapidly than C35 mice ($P < 0.01$). Initial and final weights over 40 d were 19.27 ± 4.18 and 29.40 ± 5.17 for C20, 18.92 ± 3.76 and 23.98 ± 4.67 for C35, 17.25 ± 2.65 and 23.97 ± 2.31 for S20, and 15.63 ± 2.28 and 23.48 ± 2.58 for S35, respectively. Calculated food intake, g feed per mouse per day over the study period was 3.54 ± 0 for C20, 2.84 ± 0.78 for C35, 2.85 ± 0.07 for S20, and 2.49 ± 0.29 for S35, based on the mean of 2 studies per group. As expected, there was an interaction of mouse type by diet on weight gain, $P < 0.01$ (Table 2).

Circulating CRP, IL-6, and liver mRNA expressions

S20 mice had higher plasma CRP ($P < 0.05$) and IL-6 concentrations ($P < 0.01$) than the C20 mice (Table 3). However, plasma CRP and IL-6 concentrations were lower for S35 compared with S20 mice ($P < 0.05$ for both). Diet did not affect plasma levels of CRP or IL-6 in the C group. Similarly, liver CRP mRNA expression was lower in the S35 group than in the S20 group ($P < 0.01$). S35 mice had lower levels of liver IL-6 mRNA expression than the C35 mice ($P < 0.05$) (Table 3).

Circulating arginine and liver arginase activity

We measured circulating arginine levels and liver arginase activity to obtain preliminary information about a possible mechanism for the reduction of inflammatory proteins in S35 mice. Circulating arginine levels were lower for S20 mice than for C20 mice ($P < 0.05$) (Table 3). Interestingly, the S35 mice maintained lower circulating arginine levels than C35 mice ($P < 0.05$) (Table 3). Liver arginase activity tended to be lower for S35 mice than for C35 mice ($P = 0.06$).

Discussion

The S35 mice gained more weight than the S20 mice. These results confirm that S mice have an increased need for energy from dietary protein compared with the C mice. In contrast, the C35 mice had less weight gain than the C20 group. This study supports our hypothesis and shows for the first time, to our knowledge, that increased dietary protein alone improves weight gain in the Berkeley S mouse model. The 20% protein diet inadvertently contained lower fat and sucrose than the other diets. However, there was a consistent increase in the rate of weight gain for S mice with increasing dietary protein (15, 20, 25, and 35% of energy), whereas the opposite result was observed for the C mice. Hence, we conclude that the protein:energy ratio is the major determinant of the dietary effects observed in these studies.

The significantly slower weight gain for C35 mice supports the notion that removal of a level of nitrogen that presents a toxic metabolic load to the system is occurring (33). There is evidence to suggest that at very high levels of protein consumption in control mice, the availability of some nonessential amino acids may be limited by the extent to which they are consumed in the detoxification of excess essential amino acids (33), thus limiting growth. Conversely, the high protein diet may be increasing the availability of amino acids that are limiting in sickle cell disease and required for growth and repair. Adults with HbSS are reported to have a reduced plasma essential: nonessential amino acids ratio (34), although urinary losses of amino acids are significantly reduced, compared with matched HbAA controls. Plasma arginine, leucine, valine, and histidine were among the most severely reduced essential amino acids. Both arginine and histidine are essential for growth (35). Low circulating levels of other amino acids have been documented, such as cysteine (36), the rate-limiting precursor for glutathione synthesis. Low cysteine levels in sickle cell disease may be related to low plasma pyridoxine levels (37), because pyridoxal phosphate is a required cofactor for the conversion of homocysteine to cystathionine, a precursor for cysteine synthesis.

These results also demonstrate that sickle mice fed a high protein diet at weaning had lower circulating and liver mRNA levels of the sensitive markers of inflammation, CRP, and IL-6. Activated monocytes, endothelial cells, and fibroblasts produce IL-6, which functions in both innate and adaptive immunity. IL-6 then stimulates synthesis of acute phase proteins such as CRP via hepatocytes. Both inflammatory markers are key indicators of a predominantly proinflammatory pathway, activated by other proinflammatory cytokines such as tumor necrosis factor-α and IL-1β (38). Although the cytokine profile of steady-state HbSS-mediated inflammation is still unfolding (39), this study is the first report to our knowledge of a role for antiinflammatory effects of a high protein diet in a transgenic sickle cell mouse model.

Although determining the mechanism for the reduced inflammatory proteins was not the main purpose of this study, we tested the hypothesis that the sickle cell mice had increased L-arginine availability from the high protein diet for nitric oxide production and to prevent microvascular injury. Arginine deficiency in sickle cell disease (40) is gaining interest,

particularly because arginine is a substrate for the synthesis of nitric oxide, which is a potent vasodilator. Low nitric oxide availability is considered a central feature of endothelial dysfunction and ultimate vasoocclusive crises in sickle cell disease. A shift in arginine metabolism toward enhanced urea production via the arginase pathway vs. nitric oxide production via the nitric oxide synthase pathway has been demonstrated (9,41). These observations concur with our previous findings of increased urea production rate and urea nitrogen retention in adults with homozygous sickle cell disease (5,6) and our present results suggest that a high protein diet may correct the shift observed in arginine metabolism, as demonstrated by reduced liver arginase activity in sickle cell anemia. Kaul et al. (27) recently demonstrated that arginine supplementation in sickle cell mouse models markedly increased nitric oxide generation. These results are consistent with arginine supplementation enhancing nitric oxide production in a clinical trial (42). However, the 35% energy from protein supplied in our studies did not raise arginine levels in sickle mice, just as the Comprehensive Sickle Cell Center study of 0.05–0.1 g/kg did not increase arginine levels in sickle cell patients (43), suggesting that the requirement for increased arginine is extremely high.

In summary, a high protein diet improved weight gain and reduced inflammatory proteins CRP and IL-6 in the Berkeley mouse model of sickle cell disease. We believe that the high protein diet is increasing the availability of amino acids, which have become limited in sickle cell disease, because of an increased demand on protein synthesis for red cell replacement. This increased metabolic demand is not satisfied by a diet that is adequate for healthy individuals with normal hemoglobin.

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TABLE 1

Weight gain in C57BL/6 and sickle mice fed semipurified diets containing 15–35% of energy as protein[1]

Mice	15% Protein	20% Protein	25% Protein	35% Protein
		$g \cdot g\ feed^{-1} \cdot d^{-1}$		
C	0.05 (0.04–0.06)	0.06 (0.05–0.08)	0.05 (0.04–0.06)	0.04 ** (0.03–0.06)
S	0.05 (0.04–0.06)	0.05 (0.04–0.07)	0.07 (0.06–0.08)	0.07 * (0.06–0.09)

[1] Values are geometric means (95% CI), $n = 5–8$.

* Different from C fed that diet, $P < 0.01$;

** Different from corresponding 20% protein group, $P < 0.01$ (Mann-Whitney U test).

TABLE 2

Effects of mouse type (sickle group), diet (protein level), and their interaction on weight gain[1,2]

Test	Partial SS	df	MS	F	*P* > F
Model	1.44	3	0.48	3.94	0.019
Group	0.34	1	0.34	2.78	0.107
Protein level	0.01	1	0.01	0.11	0.742
Group × protein level	1.04	1	1.04	8.56	0.007
Residual	3.29	27	0.12		

[1]Results of 2-way ANOVA on transformed data (natural log).

[2]SS, Sum of squares; df, degrees of freedom.; MS, mean square.

TABLE 3

Plasma concentrations of CRP, IL-6, and arginine and liver CRP and IL-6 mRNA expressions and arginase activity, in C57BL/6 and sickle mice fed semipurified diets containing 20 or 35% of energy as protein[1]

Group by diet	Plasma CRP	Plasma IL-6	Liver CRP mRNA	Liver IL-6 mRNA	Plasma L-arginine	Liver arginase
	μg/L	*ng/L*	*Fold-change*[2]		*μmol/L*	$\mu U \cdot g^{-1}$
C20	194 (179–211)	7.85 (5.45–11.32)	1.02 (0.93–1.11)	1.09 (0.78–1.51)	62 (33–115)	2.5 (1.9–3.2)
C35	193 (139–268)	4.66 (1.22–17.76)	0.89 (0.68–1.17)	1.16 (1.09–1.23)	56 (27–116)	4.1** (2.8–5.9)
S20	215* (197–234)	17.40* (12.24–24.72)	1.18 (1.11–1.27)	1.13 (0.90–1.43)	10* (6–17)	3.4 (1.6–7.6)
S35	200** (184–217)	6.80** (2.77–16.61)	0.92** (0.76–1.11)	1.04* (0.98–1.10)	18* (6–52)	2.7 (1.8–4.1)

[1] Values are geometric means (95% CI), $n = 3–8$.

* Different from C fed that diet, $P < 0.05$;

** Different from corresponding 20% protein group, $P < 0.05$ (Mann-Whitney U test).

[2] Normalized to 18S rRNA.