Proinflammatory Cytokines and the Hypermetabolism of Children with Sickle Cell Disease

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Sickle cell anemia (HbSS) includes chronic inflammation, but the origin is unclear. We hypothesized that in stable HbSS patients the inflammation was associated with hypermetabolism. We compared selected hypermetabolic and key immunomodulator indicators in HbSS versus control children and examined associations between measures of hypermetabolism and inflammation. Twelve fasting asymptomatic HbSS children 6–12 years and 9 controls matched for age, gender and fat mass (FM) were studied. Proportional reticulocyte count (retic%) and resting energy expenditure (REE) represented hypermetabolism, and C-reactive protein (CRP) indicated inflammation. Proinflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), chemokine monocyte chemoattractant protein-1 (MCP-1), and energy balance cytokine leptin were measured. Methods were indirect calorimetry, enzyme-linked immunosorbent assay, and radioimmunoassay. Statistical analysis included simple correlation and regression analysis. REE (51 ± 6 vs. 43 ± 12 kcal/kg per fat-free mass (FFM), mean ± SD), retic% (12 ± 4 vs. 0.7 ± 0.3%), CRP (5 ± 3 vs. 0.3 ± 0.4 mg/liter), and IL-6 (71 ± 40 vs. 20 ± 7 pg/ml) were significantly higher for HbSS than controls ($P < 0.05$). Conversely, leptin (0.1 ± 0.1 vs. 2 ± 1 µg/liter per kgFM) and MCP-1 (34 ± 5 vs. 41 ± 4 pg/ml) were significantly lower for the HbSS subjects ($P < 0.01$). TNF-α was not significantly different. There were no significant associa-

tions between REE or retic% and any cytokine measured. However, CRP was significantly associated with REE in HbSS ($r = 0.8$, $P = 0.003$) and an important predictor of REE/FFM. We provide new evidence for low circulating levels of inflammatory chemokine MCP-1 in stable HbSS children, confirm mostly low cytokine levels, inflammation, and hypermetabolism and demonstrate association of hypermetabolism with inflammation via CRP but not via cytokines. Exp Biol Med 230:68–74, 2005

Key words: energy expenditure; protein catabolism; hemolysis; chemokines

Introduction

Symptom-free children with homozygous sickle cell disease (HbSS) are hypermetabolic compared with healthy controls carrying the normal hemoglobin genotype (HbAA) (1, 2). Hypermetabolism is generally associated with cytokine driven inflammation in cases of trauma injury or infection (3, 4), but in HbSS the pattern and role of the inflammatory cytokines is unclear. Reports in the literature suggest a central role for inflammation in the disease process of HbSS patients. Elevated basal leukocyte counts (5) are typical, including activated monocytes (6) and HbSS children with the highest white blood cell counts are more likely to develop disease complications such as frequent pain and stroke (7). Acute phase reactants such as C-reactive protein (CRP) are moderately increased in stable symptom-free patients and significantly increased during painful vaso-occlusive crisis (8, 9). However, whether cytokines are involved as mediators of the inflammation of HbSS remains inconclusive, as there are only a few reports on the topic. Some investigators report elevated plasma levels of certain proinflammatory cytokines, for example, tumor necrosis factor-α (TNF-α) (10, 11), supporting a role for cytokine driven inflammation. Others report normal levels of some of the same proinflammatory cytokines (12–14) and reduced

levels of others such as interferon γ (IFN-γ) (15). However, some studies report high circulating levels of anti-inflammatory cytokines in the symptom-free state (16), the latter suggesting a potential anti-inflammatory response. Hence, no consistent pattern of cytokine involvement has been confirmed for asymptomatic HbSS patients, and furthermore, to our knowledge, no study has looked at the relationship between the hypermetabolism and proinflammatory markers in HbSS. We proposed the hypothesis that inflammation in HbSS is associated with the hypermetabolism. We measured standard indices of hypermetabolism and selected cytokines and chemokines known to mediate inflammation via leukocyte recruitment, in stable HbSS children versus healthy HbAA controls. The objectives of the study were to establish a baseline pattern of circulating cytokines in HbSS children and to investigate a possible relationship between the hypermetabolic stress and the inflammation.

Subjects and Methods

Subjects. Twelve prepubertal children (five male, seven female) with HbSS, confirmed by hemoglobin electrophoresis, were recruited for study from the Georgia Comprehensive Sickle Cell Center in Atlanta. Nine children (three male, six female) with HbAA, confirmed by sickle cell testing, and matched for age, gender, fat mass (FM), and ethnicity served as controls. The control children were recruited from the pediatric clinic at Morehouse Medical Associates in Atlanta, Georgia. All the HbSS participants were clinically asymptomatic at the time of investigation and specifically not experiencing sickle cell crisis or other complications. Children were also excluded from this study for any other medical issues that may alter protein and energy metabolism and nutritional needs: renal disease (1+ proteinuria, high creatinine level for age), abnormal liver function (ALT and serum albumin levels), chronic asthma, antisickling therapy such as hydroxyurea, blood transfusion during the preceding 4 months, and infection. Patients were also excluded if they had taken any pain medicine 3–5 days before study. Written informed assent and consent were obtained from the participants and their guardians after all aspects of the study were explained in detail. Institutional Review Boards of Morehouse School of Medicine and Emory University School of Medicine approved the study.

Study Design. The study was designed as a cross-sectional comparison study. Subjects were admitted to the study by selection of persons fitting the enrollment criteria, determined at a prestudy visit to the Emory General Clinical Research Center (GCRC). Subjects qualifying for study were admitted to the GCRC on the night before a defined experimental day for the metabolic measurements. After a 12-hr overnight fast, the study period began at about 0800 hrs and was spent in bed resting as required for measuring REE. REE was measured for 30 mins then a fasting blood sample was taken to measure hematology, reticulocyte count, plasma CRP and cytokine concentrations.

Outcome Measures for the Study. We examined uncorrected reticulocyte count (an index of erythropoietic potential) and REE as markers for hypermetabolism. CRP (a positive acute phase reactant) is a sensitive indicator of inflammation. In addition the following cytokines and chemokines were measured:

Proinflammatory cytokines TNF-α, which plays an important role in regulating inflammation and stimulates neutrophil recruitment and synthesis of many acute phase proteins; interleukin-6 (IL-6), which also stimulates synthesis of acute phase proteins; and interleukin-1β (IL-1β) which stimulates synthesis of acute phase proteins and chemokines and induces endothelial cell adhesion molecules.

Leptin (energy balance cytokine) was selected for its role in regulating energy metabolism and TNF-α release from monocytes (17).

Monocyte chemoattractant protein-1 (MCP-1), a chemokine expressed by mononuclear leukocytes, which are activated in HbSS (6), and is involved in leukocyte migration. Although it is an important chemokine mediating chronic inflammatory diseases, circulating levels of MCP-1 have not been reported for HbSS.

Regulated upon activation, normal T-cell expressed and secreted (RANTES), a chemokine also associated with leukocyte recruitment in acute and chronic inflammatory disease, but HbSS plasma levels are unknown.

Interleukin-10 (IL-10), an anti-inflammatory cytokine which is involved in limiting the inflammatory responses in some diseases.

Hematology. Automated clinical flow cytometer in the clinical hematology laboratory provided peripheral blood counts and reticulocyte and monocyte fractions by automated differential counters.

Cytokine, Chemokine, and CRP Measurements. After subjects fasted overnight (≥12 hrs) blood was drawn by venipuncture at approximately 0800 and collected in prechilled tubes containing Na_2EDTA. The plasma was separated immediately by centrifugation at 4°C and stored at −80°C until analysis. Plasma concentrations for TNF-α (detection limit 1.7 pg/ml), MCP-1 (detection limit <20 pg/ml), RANTES (detection limit <3 pg/ml), IL-10 (detection limit <1 pg/ml), IL-6 (detection limit 3 pg/ml), and IL-1β (detection limit 2 pg/ml), were measured by enzyme linked immunosorbent assay (ELISA) using standard commercially available kits (Biosource International, Inc., Camarillo, CA). ELISA also measured CRP (detection limit 0.12 ng/ml) with a commercially available kit (ALPCO Diagnostics, Windham, NH). Leptin concentrations were measured with a commercially available radioimmunoassay kit (Linco Research, St. Louis, MO) with a detection limit of 6 pg/ml (intraassay and interassay variations of <5% and <8% respectively).

Resting Energy Expenditure (REE). REE (kcal/d) was measured after subjects fasted for 12 hrs overnight. The

measurement was by indirect calorimetry over 30 mins, using a metabolic cart (DeltaTrac, SensorMedics, Yorba Linda, CA) with hood while the subjects remained supine, resting, and undisturbed. Rates of oxygen consumed (Vo_2) and carbon dioxide (Vco_2) exhaled were measured and energy expenditure was calculated using the data acquisition system of the metabolic cart.

Body Composition. Body weight was measured to the nearest 0.05 kg, using a digital scale (TANITA Corporation of America, Inc., Arlington Heights, IL) with the subjects wearing light clothing and no shoes. Height was measured with a wall mounted digital stadiometer (Heightronic, Measurement Concepts, Snoqualmie, WA) to the nearest 0.01 cm, and body mass index (BMI kg/m^2) was calculated from weight/height. Triceps and subscapular skinfold thickness (Harpenden skinfold caliper) and mid-upper arm circumference were measured by one trained observer. Percent body fat was calculated from the sum of the two skinfolds using the equations of Slaughter *et al.* (18) for children. FM and fat-free mass (FFM) were calculated from body weight.

Statistical Analysis. Results are presented as means ± SD. Student's *t* or Mann-Whitney *U* tests were used to compare normally distributed variables between groups. Spearman's correlation test was used to identify associations and regression analysis was performed. Use of non-parametric statistics was based on sample size and distribution. Group comparisons and correlation tests for leptin and REE were made after adjusting for significant predictors, FM (19) and FFM (20) respectively. Therefore leptin/FM and REE/FFM ratios were compared between the groups as customary (2, 21–22). For each statistical test outliers (defined as values >5 standard deviations from the mean) were excluded. Based on this definition, seven values were excluded. Excluding these values did not affect the conclusions obtained. Statistical significance was assumed at a $P < 0.05$ for significance tests, which were all two-tailed. SPSS version 11.0 (SPSS Inc., Chicago, IL) was used for data analysis.

Results

Subjects Characteristics. There was no significant age difference between HbSS patients and HbAA controls. The HbSS had slightly lower FFM, FM, height, and weight, but not statistically significant. When compared with the HbAA group, the mean BMI for the HbSS was significantly

lower, $P < 0.05$ (Table 1). Values for complete blood count are presented in Table 2. All HbSS patients had hemoglobin concentrations and hematocrit below the normal range and significantly lower than the HbAA group, $P < 0.001$. Circulating levels of white blood cells were twice as high for the HbSS group as for the HbAA controls, suggesting baseline inflammation for the HbSS. However, proportional monocyte count was similar for both groups (Table 2) and there was a trend toward inverse association between proportional monocyte count and MCP-1 for the HbSS, $r = -0.52$, $P = 0.08$. Compared with the HbAA controls, the HbSS patients had significantly higher proportional reticulocyte counts ($P < 0.001$), indicating potentially more rapid RBC production rate (Table 2). Reticulocyte % was significantly associated with BMI (Fig. 1) among HbAA ($r = 0.68$, $P = 0.046$) but not HbSS subjects ($r = 0.17$, $P = 0.64$).

REE, Plasma Cytokines and CRP. Figure 2 shows REE, selected plasma cytokines and CRP concentrations for individual subjects. As expected, REE was significantly associated with FFM across both groups ($r = 0.61$, $P = 0.007$) and REE adjusted for FFM was significantly higher for the HbSS children ($P < 0.005$). Mean MCP-1, IL-10 and leptin/FM were significantly lower for the HbSS children compared with the controls ($P < 0.01$). Conversely, CRP and IL-6 were significantly higher for the HbSS group ($P < 0.05$). TNF-α (54 ± 7 vs. 50 ± 4) RANTES (9 ± 4 vs. 7 ± 5) and IL-1 β (100 ± 82 vs. 100 ± 76) were not different when HbSS and HbAA subjects were compared. Although leptin concentration was significantly lower and REE significantly higher for the HbSS group versus controls, leptin was not correlated with REE in the HbSS ($r = 0.04$, $P = 0.92$). For neither group were MCP-1 and reticulocytes correlated, HbSS ($r = -0.01$, $P = 0.98$) and HbAA ($r = -0.04$, $P = 0.92$). However, CRP was significantly correlated with REE (Fig. 3) in the HbSS group ($r = 0.80$, $P = 0.003$), but not the HbAA ($r = 0.26$, $P = 0.62$). Furthermore, regression analysis demonstrated that CRP was an important predictor of corrected REE in the HbSS ($r^2 = 0.64$, $P < 0.005$), B = 1.88 for CRP. CRP was still a significant predictor of REE after adjusting for age ($r^2 = 0.74$, $P = 0.012$), FM or gender ($r^2 = 0.65$, $P = 0.007$), but not BMI ($r^2 = 0.48$, $P = 0.06$). When CRP, age, FM, gender and BMI were included in the model together, age (B = -1.48, $P = 0.04$) was the only significant predictor of REE and for the model $r^2 = 0.91$, adjusted $r^2 = 0.75$. Using stepwise regression analysis among HbSS only, age was the

Table 1. Age and Body Composition in HbAA and HbSS Subjects[a]

Subject (n)	Age, years	FFM, kg	FM, kg	Weight, kg	Height, cm	BMI, kg/m²
HbAA (9)	9 ± 2	29 ± 9	6 ± 2	35 ± 10	139 ± 18	18 ± 2
HbSS (12)	9 ± 2	24 ± 6	4 ± 2	28 ± 7	133 ± 12	16 ± 1
P	0.462	0.254	0.088	0.176	0.569	0.006

[a] Values are mean ± SD; P values Mann-Whitney U test. FFM = fat-free mass, FM = fat mass, BMI = body mass index. BMI was significantly lower for the HbSS (sickle cell disease) group compared with the HbAA (normal) group.

Table 2. Comparison of Hematological Measurements for HbAA and HbSS Subjects[a]

Subject (n)	Hb g/liter	HCT %	WBC	Monocyte, %	RBC	Retic, %
Normal range	110–174	33–52	3.7–9.4 × 10^9/liter	5–12	3.6–5.8 × 10^{12}/liter	0.5–2.0
HbAA (9)	124 ± 8	37 ± 2	6.0 ± 0.7	8 ± 3	4.4 ± 0.3	0.7 ± 0.3
HbSS (12)	84 ± 13	24 ± 4	12.7 ± 3.4	9 ± 3	2.8 ± 0.5	11.5 ± 4.0
P	<0.001	<0.001	<0.001	0.347	<0.001	<0.001

[a] Values are mean ± SD, P values Student's t test. Hb = hemoglobin, HCT = hematocrit, WBC = white blood cell, RBC = red blood cell, Retic = reticulocyte. HbSS had significantly lower Hb, HCT and RBC and significantly higher WBC and Retic% compared with the HbAA. Monocyte % was not different across the groups.

best predictor of REE (r^2 = 0.71, B= −1.55, P < 0.005) and combining the two study groups, both age (B = −0.78) and CRP (B = 1.65) could predict REE (r^2 = 0.80, P < 0.001). Other significant correlations observed were among the HbAA group, leptin versus MCP-1 (r = 0.67, P = 0.05), TNF-α versus MCP-1 (r = 0.74, P = 0.02) and among the HbSS group, MCP-1 versus IL-1β (r = −0.70, P = 0.02).

Discussion

The characteristic response to stresses of trauma injury, infection, and other disease, includes hypermetabolism and protein catabolism (3, 4, 23–24) associated with a cytokine-

y = 0.129x - 1.538, r = 0.68, P = 0.046

Figure 1. Correlation of body mass index (BMI) and reticulocyte count (retic%) among the HbAA controls. BMI was significantly associated with retic%, P < 0.05.

driven inflammatory response in which increased levels of cytokines and acute phase proteins are considered to induce the hypermetabolism. Stable HbSS patients portray many similar characteristics including elevated energy expenditure, protein turnover (1, 2, 25–27), protein catabolism via increased urea production rate (28–29), increased plasma concentrations of acute phase proteins (8) and leukocytosis (5). However, the results of the present study demonstrate an unusual inflammatory profile, with no direct associations of inflammatory cytokines with the hypermetabolism measured. The HbSS children had an average increased REE per FFM of 19% compared with the HbAA controls, similar to previous reports with larger sample size (2, 22). Reticulocyte count was also 16 times higher for the HbSS, indicating a more rapid rate of erythropoiesis and mean CRP concentration and white blood cell count were significantly elevated.

Increased leukocyte aggregation is usually mediated by proinflammatory chemokines, including MCP-1 and RANTES (30) in response to inflammatory cytokines (e.g., TNF-α and IFN-γ). However, in the present study TNF-α was not significantly different for the HbSS children compared with the HbAA controls, similar to previous reports (13). As far as we are aware this study is the first to report circulating levels of MCP-1 in HbSS, which were significantly lower than for the HbAA controls, although others have reported increased MCP-1 mRNA from monocytes in steady state sickle cell patients (31). We are currently investigating this and other pathways in a mouse model of HbSS (32), to determine why RNA message may not translate into functional protein. These HbSS patients also had significantly lower leptin concentrations, similar to a previous report with larger sample size in adolescents and adults (33), but neither MCP-1 nor leptin (33) was associated with the hypermetabolic markers (REE and reticulocyte count), although CRP concentration was associated with REE in HbSS.

Interestingly, our expectation of increased proinflammatory protein expression as a feature of the HbSS mediated inflammation was not realized. Based on this result we also measured proinflammatory chemotactic protein RANTES with potent leukocyte recruiting activity similar to MCP-1, IL-6 and IL-1β which stimulate synthesis of acute phase proteins and anti-inflammatory cytokine IL-10. The RANTES results were variable, most likely because of
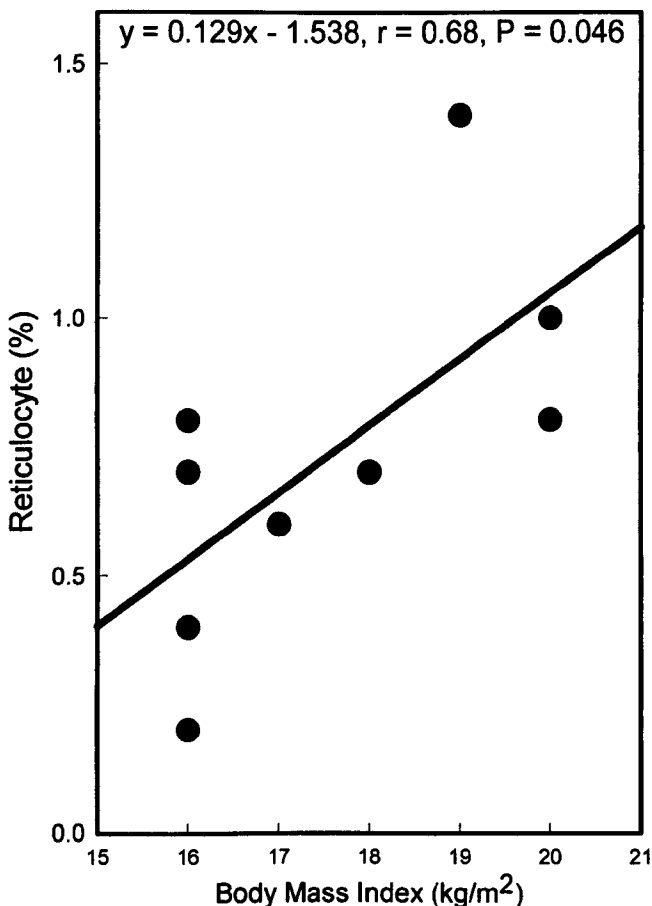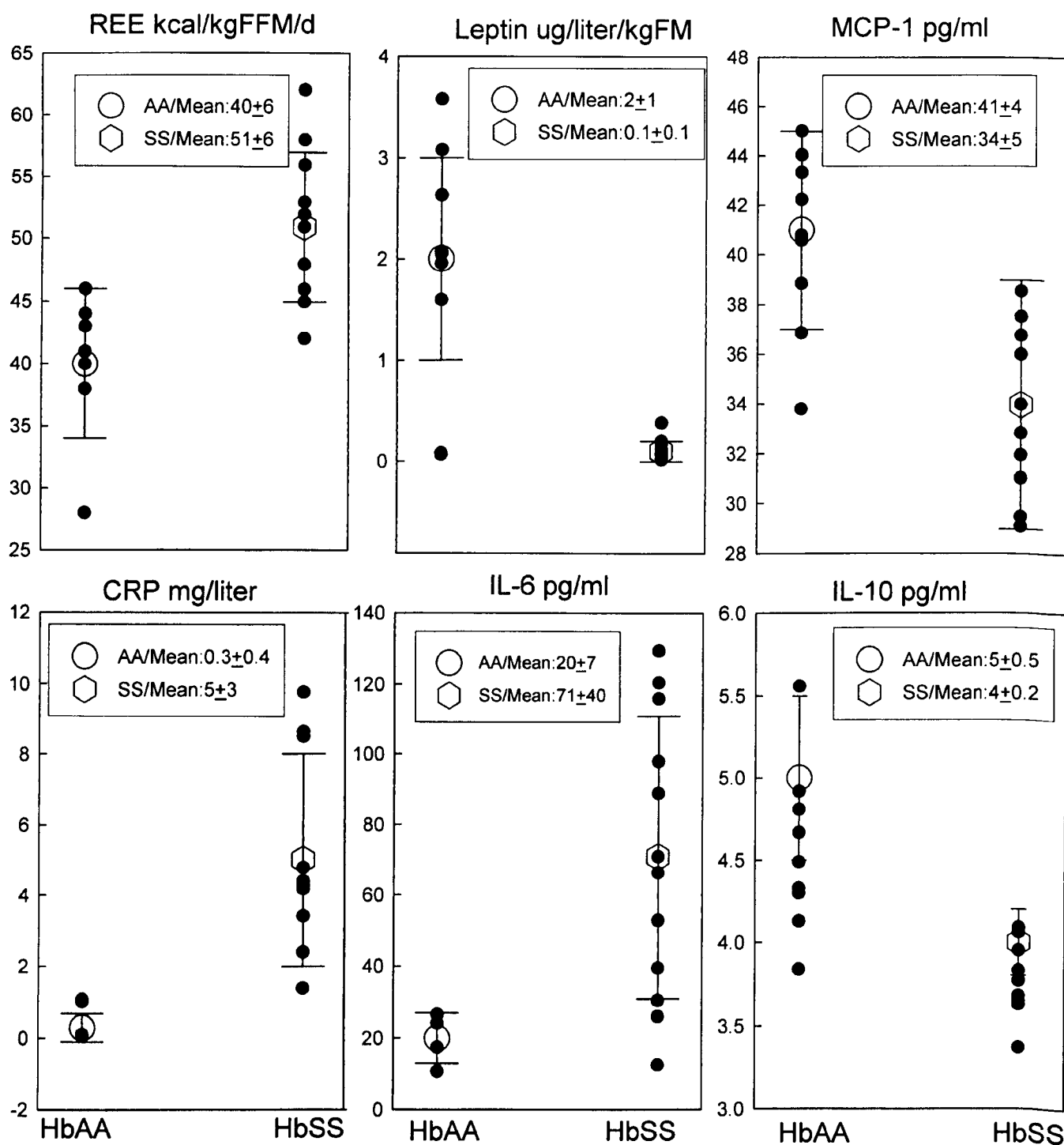
Figure 2. Comparison of resting energy expenditure and selected plasma cytokines for HbAA and HbSS subjects. Values are mean ± SD, P values Mann-Whitney U Test. Group comparisons tests for resting energy expenditure (REE) and leptin were made after adjusting for significant predictors, fat-free mass (FFM) and fat mass (FM), respectively. MCP = monocyte chemoattractant protein, CRP = C-reactive protein, IL = interleukin. HbSS had significantly increased REE (P = 0.001), CPR (P < 0.001) and interleukin 6 (P = 0.013) compared with the HbAA. Leptin (P = 0.003), MCP-1 (P = 0.006) and interleukin-10 (P < 0.001) were significantly lower for the HbSS, whereas tumor necrosis factor-α, regulated upon activation, normal T-cell expressed and secreted (RANTES), and interleukin-1β, not shown, were not different across the groups.

increased platelet damage common in HbSS, with no mean difference between the groups. There are no known reports of circulating RANTES in the HbSS literature for comparison. IL-1β was not different for the groups in this study, but IL-6 was significantly higher for the HbSS. Other researchers (14, 34) have reported similar results. IL-10 was significantly lower for HbSS than HbAA subjects, similar to other findings of no detectable IL-10 in HbSS (35). Together these findings suggest no significant involvement of IL-10 mediated anti-inflammatory response in children
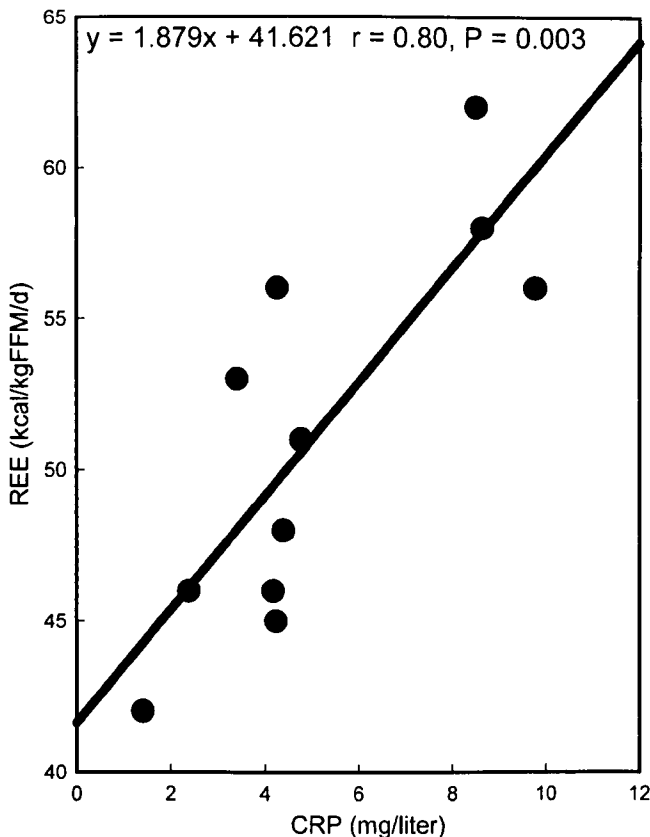
Figure 3. Correlation of resting energy expenditure (REE) versus C-reactive protein (CRP) for the HbSS group. The correlation test was done after adjusting REE for the major significant predictor, fat-free mass (FFM). CRP was significantly associated with REE, $P < 0.005$. Furthermore, regression analysis indicated CRP to be an important predictor of REE in HbSS ($r^2 = 0.64$, $P < 0.005$).

with HbSS. An alternative explanation could be occurrence of localized high concentrations of MCP-1, TNF-α, and IL-10 that do not circulate in plasma, yet promoting leukocyte activation by paracrine effects. The high levels of the rapidly responding acute phase protein CRP (36) support the idea that repeated short duration stimuli, such as sub-clinical ischaemic episodes (37), promote inflammation in symptom-free sickle cell disease. The strong association of CRP and REE hint at a link between inflammation and hypermetabolism via acute phase proteins.

The results of this study confirm hypermetabolism in this group of asymptomatic HbSS children related to chronic covert inflammation demonstrated by both elevated CRP levels and baseline leukocyte recruitment. However, the surfeit of leukocytes may not express many of the expected proinflammatory cytokines (except for IL-6) or chemokines even during acute vaso-occlusive crisis (38). Other mechanisms or pathways may regulate the inflammation or the cytokines and chemokines may be localized and not released into the plasma. Altogether, our observations of significantly reduced MCP-1, leptin and IL-10, significantly increased CRP and IL-6, plus published reports of reduced IFN-γ reveal a unique immunomodulator profile that may

represent a sum of compensatory mechanisms to control possible harmful effects of chronic hyper-inflammation.

There are some practical concerns limiting our ability to fully interpret these results. Although we are aware of temporal changes of cytokines/chemokines, this study was designed only as a cross sectional comparison of these markers in stable HbSS patients and healthy controls, to establish disease-related alterations. This was the most practical approach because chronic leukocytosis is typical of this disease, hence it is difficult to examine the temporal changes at baseline in the same way as inflammation produced by an acute infection or trauma injury. Some differences, particularly leptin, may have been influenced by the significantly different BMI, since the controls were chosen mainly for matching fat mass because of difficulty matching for both fat mass and BMI within a single age group in these prepubertal children. However, BMI did not correlate with leptin or any other cytokine in this study. Moreover, a more comprehensive list of cytokines might give a better picture of this response to inflammation in stable HbSS. It is difficult to choose all the factors pertinent to HbSS without a global picture such as gene expression to start, which we are presently investigating using the mouse model previously mentioned (32) that expresses uniquely human sickle cell anemia.

This study provides new important findings of low circulating levels of chemotactic cytokine MCP-1, despite the chronic leukocytosis of HbSS pathology and CRP as an important predictor of REE. A predictive value of CRP for REE has previously been reported in cystic fibrosis (39) and AIDS patients (40). More detailed investigations of the role of alternative pathways in this HbSS related inflammation are needed. Availability of transgenic sickle mice provides a useful model for such studies (32, 41). These studies will be of critical importance in seeking more effective therapeutic modalities for sickle cell disease such as methods for reducing sub-clinical tissue injury.

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