Clinical Observations, Plasma Retinol Concentrations, and In Vitro Lymphocyte Functions in Children With Sickle Cell Disease

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Background: Children with sickle cell disease (SCD) often have infections, growth deficits, and impaired immunity, problems that also are observed in individuals with a vitamin A deficiency (plasma retinol concentration <20 μg/dL). The goal of this study was to investigate the association between vitamin A, health status, and the in vitro immune function of children with SCD.

Methods: Fifty-nine children (40 SS, 11 SC, and 8 Sβthalassemia [Sβthal] hemoglobin genotypes) 9 months to 18 years old were investigated for plasma levels of retinol, retinol binding protein, C-reactive protein, alpha-1-acid glycoprotein, lymphocyte proliferation, and interleukin (IL)-2 activity in supernatant of phytohemagglutinin-treated lymphocytes.

Results: The plasma retinol concentrations of children with SCD (mean 57.6 μg/dL, range 4.6-116 μg/dL) were not different from those of 21 normal individuals (mean 62 μg/dL, range 28.7-162 μg/dL). Plasma retinol concentrations did not vary by hemoglobin genotype but were lower in boys than in girls (P < 0.05) and were also lower in children with inflammation (P = 0.1). Seven children (11.9%) (6 HbSS, 1 HbSβthal) were vitamin A–deficient, and 9 children (15.3%) had suboptimal vitamin A status (plasma retinol concentration of 20-29 μg/dL). Children with vitamin A deficiency had slightly lower height (P = 0.09) and weight mean percentiles, lymphocyte proliferative responses, and IL-2 activity (P > 0.1), but higher means of C-reactive protein (P = 0.05), pain crisis episodes and inflammation (P = 0.1), and health scores (P > 0.1) than children who were not vitamin A–deficient. Lymphocyte proliferative responses negatively correlated with health score, pain crisis episodes, and blood units received, but positively correlated with retinol binding protein (P < 0.05 to P = 0.1).

Conclusion: Identification and correction of suboptimal vitamin A status in children with SCD may improve immunity and attenuate certain health complications associated with this disease.

Keywords: Anemia–sickle cell, infection, inflammation, interleukin-2, lymphocyte proliferation, vitamin A

INTRODUCTION

The role of vitamin A in morbidity and mortality resulting from infectious illnesses in humans and laboratory animals has been well established.1,2 Vitamin A deficiency (plasma retinol <20 μg/dL) impairs antibody responses to T cell–dependent antigens and immunoglobulin (Ig) M switching to IgA, decreases the percentage of Th2+ cells, impairs T cell homing in the gut (one of the main routes of pathogen entry into the body), increases gut permeability to pathogens and antigens, and increases susceptibility to infections.3,7 Increased susceptibility to infection reduces growth rate, either through nutrient loss or by reduced food intake. Vitamin A deficiency also induces a proinflammatory response with elevated blood levels of type 1 cytokines such as tumor necrosis factor-alpha (TNF-α) and interferon gamma but low anti-inflammatory cytokines such as interleukin (IL)-4 and IL-10.2,3

In a previous study involving 34 children with sickle cell disease (SCD), we observed that almost one in every two children had retinol binding protein <30 mg/L.8 In contrast, only 20% of children had a transferrin concentration <2 g/L, and no child had albumin levels <35 g/L. The data suggested that suboptimal vitamin A status (as suggested by low plasma retinol binding protein concentration) rather than general protein energy malnutrition might have been responsible for the low retinol binding protein concentrations.

Growth retardation (low body weight for age, height for age, body fat, and delayed skeletal maturation) is common in children with SCD.2-12 The mechanisms of growth retardation are likely multifactorial and may involve deficits...
in protein, energy, and micronutrients such as zinc and vitamin A.\textsuperscript{13-16}

Although numerous studies have been conducted on the effect of deficits or supplementation of certain micronutrients, specifically zinc, on in vitro immune function and infection rates in children with SCD, there is a paucity of data on vitamin A status in the same population.\textsuperscript{13-15} This study was designed to investigate (1) vitamin A status in children with SCD who present to Children’s Hospital of New Orleans; (2) whether suboptimal vitamin A status is associated with poor clinical status and growth deficits and with altered in vitro immune responses of peripheral blood mononuclear cells in these patients; (3) whether hemoglobin genotypes, inflammation, and/or sex are confounding factors in impaired in vitro lymphocyte function associated with suboptimal vitamin A status in this population.

\section*{METHODS}

\subsection*{Study Population and Design}

The study was approved by the Louisiana State University Health Sciences Center (LSUHSC) Institutional Review Board and Children’s Hospital of New Orleans (Protocol #1789). Children whose data are included in this analysis were enrolled in a prospective study on the association between nutritional status and lymphocyte proliferation and certain complications usually associated with SCD. Inclusion criteria were (1) patients were registered at Children’s Hospital of New Orleans and received primary healthcare services related to SCD from the LSUHSC hematology/oncology physicians; (2) age 0.5 to 18 years; (3) boys and girls with HbSS, HbSC, and/or HbS\textsuperscript{thalamassemia} (HbS\textsuperscript{thal}) genotypes; and (4) patients who had not received bone marrow transplantation to correct hemoglobinopathy, splenectomy, or other major surgery related and unrelated to SCD. Patients who did not fulfill the above criteria were excluded. Prior to 1997, hydroxyurea was not prescribed to children with SCD at Children’s Hospital of New Orleans, and none of the patients had received this drug.

A letter of invitation to participate in the study was sent to parents and guardians of all potential patients that briefly described the goal of the study. Additionally, parents/guardians were briefed during clinic visits. Among the 90 children who signed a consent form, 31 children were excluded from the analysis because of missing data on at least 3 measurements (markers of inflammation, biochemical markers of nutritional status, or plasma retinol concentration).

\subsection*{Blood Drawing and Chart Review}

During clinic visits, blood samples (2-4 mL) were drawn in heparinized vacutainers under stable conditions from 59 children with SCD who were 9 months to 18 years old. Two hundred microliters (200 μL) were removed for measurement of hemoglobin, hematocrit, and white blood cell counts by standard techniques. The remaining blood samples were centrifuged at 2,000 rpm at room temperature for 10 minutes. Plasma was aspirated and aliquoted in 500 μL volumes and immediately frozen at −70°C until used for various measurements. Plasma retinol was measured by fluorescence spectrometry. For quality control, plasma retinol also was measured in frozen plasma samples obtained from individuals without SCD (7 LSUHSC laboratory employees [4 males and 3 females, age range 38-46 years, mean 43 years] and 14 children [8 males and 6 females, age range 3-18 years, mean 8.5 years]).

Information on infections, pain crises, anthropometry weight, height, age, hemoglobin genotype, and blood transfusions was obtained from patients’ charts for the period January 1994 through December 1996. To rule out the possibility that abnormal plasma retinol levels were attributable to inflammation, C-reactive protein (CRP) and alpha-1-acid glycoprotein (AGP) were measured by radial immunodiffusion, and TNF-α was measured by enzyme-linked immunosorbent assay. Inflammation was considered present when AGP and/or CRP were >1 g/L and >10 mg/L, respectively. Retinol binding protein was also measured by radial immunodiffusion because its plasma levels are reduced by vitamin A deficiency independently of inflammation and overt protein-energy malnutrition, and levels <30 mg/L are considered abnormal.\textsuperscript{17,18}

Growth deficits were present when either weight or height was below the 10\textsuperscript{th} percentile. Health scores were defined as 0 (no pain crisis episodes, infections, hospital admissions, or blood transfusions during the study period) or ≥1 (the sum of pain crisis episodes, infections, hospital admissions, and blood transfusions received during the study period).

\subsection*{Lymphocyte Proliferation}

Mononuclear cells were isolated by density gradient centrifugation on Ficoll-Hypaque from blood samples collected in heparinized blood. Cell viability was determined by trypan blue exclusion test. Lymphocyte proliferation was assessed by incubating 0.20 × 10\textsuperscript{6} viable cells per 200 μL of culture medium in the presence of 0-15 μg/mL phytohemagglutinin (PHA) at 37°C, 5% CO\textsubscript{2} in a humidified atmosphere. Per liter of RPMI-1640, the culture medium contained 100 mL heat-inactivated human AB serum, 5 × 10\textsuperscript{4} units penicillin, 50 mg streptomycin, 1 mmol sodium pyruvate, 0.1 mmol nonessential amino acids, 2 mmol L-glutamine, and 50 μmol β-mercaptoethanol. After 48 hours of incubation, cultures (made in triplicate) were pulsed with 1 μCi \textsuperscript{3}H-thymidine and further incubated for 24 hours under identical conditions. The rate of cell proliferation was assessed by estimating the amount of radioactivity uptake (incorporated into DNA expressed as counts per minute) in a liquid scintillation counter as we previously reported.\textsuperscript{19}

\subsection*{Interleukin-2 Study}

Macrophages containing 2 × 10\textsuperscript{6} viable cells were mixed with 0-15 μg PHA in a total volume of 1 mL, transferred to round bottom 12 × 75 mm culture tubes, and incubated in identical conditions as microwell cultures. After 48 hours, the tubes were centrifuged at 400 times gravity for 10 minutes. The supernatant was collected, aliquoted in 500 μL samples, and immediately frozen at −70°C. The IL-2 activity in the supernatant was assessed by the growth of the IL-2-dependent CTLL-2 cell line as described in the literature.\textsuperscript{20}

\section*{Statistical Analysis}

Descriptive statistics, analysis of variance, Pearson correlation coefficient, and chi-square test were performed with MYSTAT, second edition (SYSTAT Software, Inc.).\textsuperscript{21} Vitamin A status, IL-2 concentrations, and lymphocyte proliferative responses to PHA were analyzed as a function of sex, retinol binding protein, inflammation, and health scores. Multiple
regression analysis was computed with IL-2 and/or lymphocyte proliferation as dependent variables and health status (plasma retinol concentration, weight, height, infection, blood transfusion, acute phase proteins, retinol binding protein, hemoglobin genotype, and hematologic measurements) as independent variables. The level of significance was set at \( P < 0.05 \).

**RESULTS**

**Assessment of Clinical Status**

The mean ± SEM age of the 59 study subjects was 7.63 ± 0.59 years. The cohort included 34 boys and 25 girls. As shown in Table 1, 67.8% of patients had HbSS genotype. Children with HbSC genotype were younger than girls. As shown in Table 1, 67.8% of patients had HbSS genotype, and hematologic measurements showed no significant differences between the 2 groups of children with SCD.

Among the 7 patients with plasma retinol levels below normal, 5 boys and 1 girl had HbSS genotype, and the seventh child was a boy with HbS\( ^{\beta+} \)thal genotype. When the 5 boys with plasma retinol concentrations below normal and HbSS genotype were compared with 18 boys having the same hemoglobin genotype but with plasma retinol within the normal range, we again observed the following trend: higher mean CRP levels \( (P = 0.08) \) and inflammatory score \( (P = 0.1) \). No significant differences in the means of hematologic measurements and TNF-\( \alpha \) concentrations were observed among vitamin A–deficient and vitamin A–adequate children.

Because only 1 girl had plasma retinol below normal, statistical analysis of various measurements could not be performed as a function of sex and vitamin A status. However, in the overall study population, girls had higher mean plasma retinol concentrations than boys (Table 4, \( P < 0.05 \)). The means for weight and height percentiles tended to be higher in girls than boys, but mean infection episodes, inflammation scores \( (P = 0.07) \) (Table 3). However, all other measurements showed no significant differences between the 2 groups of children with SCD.

As shown in Figure 1, a higher percentage of children with plasma retinol below normal had at least one episode of pain crisis \( (P = 0.09) \), or had weight below the 10th percentile \( (P = 0.1) \) compared to children with concentrations within the normal range. However, the percentages of children with SCD who had white blood cell counts >15 \( \times 10^6 \) mL did not differ by plasma retinol concentrations (data not shown).

### Table 1. Demographic Characteristics of the Study Population

| Hemoglobin Genotype | HbSS | HbSC | HbS\( ^{\beta+} \)thal | Total | Mean Age, years, ± SEM |
|---------------------|------|------|------------------------|-------|-----------------------|
| Boys                | 23   | 7    | 4                      | 34    | 7.63 ± 0.71           |
| Girls               | 17   | 4    | 4                      | 25    | 7.63 ± 1.00           |
| Total               | 40   | 11   | 8                      | 59    | 7.63 ± 0.59           |
| Mean age, years, ± SEM | 8.20 ± 0.66\( ^a \) | 5.03 ± 1.24\( ^b \) | 8.34 ± 2.02\( ^b \) | 7.63 ± 0.59 |

Note: Two boys and one girl had HbS\( ^{\beta+} \)thal genotype. The mean ± SEM ages in years of children with HbS\( ^{\beta+} \) (\( n = 3 \)) and HbS\( ^{\beta+} \) (\( n = 5 \)) were 9.17 ± 2.41 and 7.85 ± 5.43, respectively (and were not significantly different). Children with HbSC genotype were younger than those with HbSS or HbS\( ^{\beta+} \)thal (\( a < b; P < 0.05 \)).

### Table 2. Plasma Retinol Levels in Patients and Controls

| Group                        | n   | Mean (± SEM) Plasma Retinol Level, \( \mu g/dL \) | Plasma Retinol Range, \( \mu g/dL \) |
|------------------------------|-----|-----------------------------------------------|-----------------------------------|
| Children with HbSS          | 40  | 57.1 ± 4.7                                   | 6-116                             |
| Children with HbSC          | 11  | 60.9 ± 5.7                                   | 39-87                             |
| Children with HbS\( ^{\beta+} \)thal | 8   | 55.5 ± 12.8                                 | 4.6-109                           |
| Children in the control group | 14  | 59.0 ± 5.88                                 | 28.7-119                          |
| Laboratory employees        | 7   | 70.0 ± 17.39                                 | 43.0-162                          |
| All patients                | 59  | 57.6 ± 3.7                                   | 4.6-116                           |
| All controls                | 21  | 62 ± 6.5                                    | 28.7-162                          |

Note: No significant differences were observed between patients and the combined controls and/or by hemoglobin genotype.

None of the plasma samples obtained from laboratory employees or from children without SCD had plasma retinol concentrations below normal. Children with plasma retinol concentrations below normal tended to have lower means for height percentile \( (P = 0.09) \) and weight percentile \( (P > 0.1) \) but higher means of pain crisis episodes \( (P = 0.05) \), CRP \( (P = 0.05) \), and inflammation scores \( (P = 0.07) \) (Table 3).
Table 3. Mean (± SEM) Age and Clinical Status of Children With Sickle Cell Disease as a Function of Plasma Retinol Concentration

| Variable                  | Plasma Retinol <20 μg/dL (n = 7) | Plasma Retinol ≥20 μg/dL (n = 52) | P Value | Plasma Retinol <20 μg/dL (n = 5 boys with HbSS) | Plasma Retinol ≥20 μg/dL (n = 18 boys with HbSS) | P Value |
|---------------------------|----------------------------------|-----------------------------------|---------|-----------------------------------------------|--------------------------------------------------|---------|
| Age, years                | 8.81 ± 1.1                       | 7.47 ± 0.65                       |         | 8.87 ± 1.51                                  | 7.22 ± 0.80                                      |         |
| Weight, kg                | 26.83 ± 2.82                     | 25.54 ± 2.33                      |         | 25.38 ± 3.35                                  | 22.53 ± 2.25                                     |         |
| Weight percentile         | 28.21 ± 10.77                    | 37.72 ± 4.62                      |         | 20.50 ± 9.25                                  | 23.06 ± 5.89                                     |         |
| Height, cm                | 131.5 ± 6.22                     | 119.26 ± 4.45                     |         | 128.8 ± 7.52                                  | 118.64 ± 6.99                                    |         |
| Height percentile         | 22.5 ± 5.82                      | 40.28 ± 4.87                      | 0.09    | 25.07 ± 6.61                                  | 26.31 ± 7.46                                     |         |
| Pain crises               | 1.57 ± 0.43                      | 0.72 ± 0.19                       | 0.05    | 1.40 ± 0.51                                  | 0.82 ± 0.23                                     |         |
| Units of blood            | 0.71 ± 0.71                      | 1.17 ± 0.33                       |         | 1.00 ± 0.998                                  | 1.35 ± 0.64                                     |         |
| Infections                | 0.57 ± 0.297                     | 0.58 ± 0.13                       |         | 0.60 ± 0.399                                  | 0.81 ± 0.28                                     |         |
| Hospitalizations          | 1.86 ± 0.96                      | 1.43 ± 0.27                       |         | 1.20 ± 0.582                                  | 2.07 ± 0.59                                     |         |
| Hematocrit, %             | 26.5 ± 1.87                      | 27.78 ± 0.69                      |         | 25.10 ± 2.28                                  | 24.22 ± 0.78                                     |         |
| Hemoglobin, g/dL          | 8.50 ± 0.54                      | 8.79 ± 0.2                        |         | 8.22 ± 0.64                                  | 7.74 ± 0.18                                     |         |
| White blood cells × 10⁶/mL| 12.10 ± 0.97                     | 12.69 ± 0.75                      |         | 11.57 ± 1.11                                  | 14.57 ± 1.26                                     |         |
| C-reactive protein, mg/L  | 18.27 ± 12.8                     | 5.50 ± 1.64                       | 0.05    | 25.58 ± 17.23                                  | 8.38 ± 4.24                                     | 0.08    |
| Alpha-1-acid glycoprotein, g/L | 0.71 ± 0.19         | 0.69 ± 0.04                       |         | 0.83 ± 0.22                                  | 0.66 ± 0.08                                     |         |
| Retinol binding protein, mg/L | 19.30 ± 4.06       | 21.56 ± 1.14                      |         | 14.72 ± 2.76                                  | 19.22 ± 1.81                                     |         |
| Tumor necrosis factor-α, pg/mL | 32.71 ± 19.7        | 50.61 ± 13.64                     |         | 33.74 ± 28.05                                  | 69.73 ± 28.62                                    |         |
| Inflammation score        | 0.71 ± 0.29                      | 0.35 ± 0.08                       | 0.07    | 0.80 ± 0.37                                  | 0.33 ± 0.16                                     | 0.1     |
| Health scoreb             | 5.43 ± 1.67                      | 3.84 ± 0.61                       |         | 5.20 ± 1.83                                  | 4.83 ± 1.09                                     |         |

*All hemoglobin phenotypes included.

*bHealth score is the sum of the number of episodes of pain crisis, infection, hospital admission, and/or blood units received.

29.3%) in children with plasma retinol concentrations below normal compared to those with levels within the normal range in both the age-adjusted and age-unadjusted analyses (P > 0.1). The same trend was observed when the analysis included only boys with HbSS genotype with plasma retinol levels <20 μg/dL vs ≥20 μg/dL (Figure 2B, P > 0.1). The mean stimulation indexes of cell cultures from children with plasma retinol concentrations and retinol binding protein below normal were 18.2% lower than those of children with normal concentrations of plasma retinol but retinol binding protein below normal level (Figure 2C, P > 0.1). Mean lymphocyte proliferative responses of peripheral blood mononuclear cells obtained from children with inflammation were 12% lower than those of children without inflammation, although differences were not statistically significant (graph not shown). A dose-dependent decrease (18.2% to 57.6%) in the rate of DNA synthesis or ³H-thymidine uptake expressed as counts per minute in PHA-treated cells, corrected for baseline counts per minute, was seen with increased health scores (Figure 2D, P < 0.05 to P = 0.1).

**Interleukin-2 Concentration as a Function of Plasma Retinol, Sex, Inflammation, and Health Scores**

In nonactivated cultures, the mean IL-2 concentrations were higher in cultures of peripheral blood mononuclear cells obtained from children with plasma retinol below normal compared to those with normal concentrations (Figure 3A, P < 0.05). In cells treated with 2.5, 5, and 10 μg/mL PHA, the mean IL-2 concentrations were 11%-26.6% lower in children with plasma retinol concentrations below normal than in those with normal concentrations (P > 0.1).

Contrary to what we expected, the mean IL-2 activity of cells treated with 15 μg/mL PHA was marginally higher for children with plasma retinol below normal than in children with plasma retinol ≥20 μg/mL.

Mean IL-2 concentrations in PHA-treated cultures from boys with HbSS genotype and plasma retinol below normal levels were 10.6% lower than those of boys with HbSS and normal plasma retinol concentrations; however, the differences were not statistically significant (Figure 3B).

In nonactivated cells, the mean IL-2 concentration of nonactivated cells obtained from children with plasma retinol and retinol binding protein below normal levels tended to be higher than that obtained from children with plasma retinol but retinol binding protein within the normal range (Figure 3C, P = 0.05). However, as expected, the mean IL-2 concentrations of PHA-treated cells obtained from children with plasma retinol and retinol binding protein below normal levels were 8.6%-27.6% lower than those of children with normal plasma retinol but retinol binding protein below normal (Figure 3C, P = 0.1 for 5 μg/mL PHA). When plasma retinol concentrations were not taken into account, we observed slightly higher (mean ± SD) IL-2 activity in cells incubated with 5 μg/mL PHA from girls (6.43 ± 2.712 U/mL) than from boys (4.478 ± 2.25 U/mL).
Table 4. Mean (± SEM) Age and Clinical Status of Boys and Girls With Sickle Cell Disease Independent of Vitamin A Status

| Variable                        | Boys (n = 34) | Girls (n = 25) |
|---------------------------------|--------------|---------------|
| Age, years                      | 6.62 ± 0.71  | 7.63 ± 1.01   |
| Plasma retinol, μg/dL           | 49.8 ± 4.90  | 68.1 ± 5.1    |
| Weight, kg                      | 25.78 ± 2.65 | 25.64 ± 3.26  |
| Weight percentile               | 34.05 ± 5.59 | 39.7 ± 6.60   |
| Height, cm                      | 121.32 ± 4.76| 119.86 ± 7.27 |
| Height percentile               | 35.76 ± 5.95 | 41.66 ± 6.65  |
| Pain crises                     | 0.72 ± 0.16  | 1.00 ± 0.38   |
| Units of blood                  | 0.91 ± 0.37  | 1.41 ± 0.49   |
| Infections                      | 0.66 ± 0.16  | 0.50 ± 0.18   |
| Hospitalizations                | 1.43 ± 0.33  | 1.57 ± 0.47   |
| Hematocrit, %                   | 26.98 ± 0.93 | 28.48 ± 0.87  |
| Hemoglobin, g/dL                | 8.58 ± 0.32  | 9.00 ± 0.36   |
| White blood cells × 10⁶/mL       | 13.30 ± 0.84 | 11.72 ± 1.06  |
| C-reactive protein, μg/mL       | 8.85 ± 0.39  | 4.44 ± 1.2    |
| Alpha-1-acid glycoprotein, g/L  | 0.689 ± 0.06 | 0.689 ± 0.05  |
| Retinol binding protein, mg/L   | 21.02 ± 1.52 | 21.63 ± 1.64  |
| Tumor necrosis factor alpha, pg/mL | 59.4 ± 17.89 | 32.55 ± 14.04 |
| Inflammation score              | 0.412 ± 0.12 | 0.36 ± 0.098  |
| Health score b                  | 3.82 ± 0.65  | 4.35 ± 1.05   |

Table 5. Mean (± SEM) Age and Clinical Status of Children With Sickle Cell Disease as a Function of Inflammation

| Variable                        | Without Inflammation (n = 40) | With Inflammation* (n = 19) |
|---------------------------------|-------------------------------|-----------------------------|
| Age, years                      | 8.354 ± 0.753                 | 6.09 ± 0.811                |
| Plasma retinol, μg/dL           | 60.9 ± 7.0                    | 50.90 ± 4.3                |
| Weight, kg                      | 28.39 ± 2.58                  | 18.864 ± 2.21              |
| Weight percentile               | 38.08 ± 5.16                  | 32.393 ± 7.63              |
| Height, cm                      | 125.64 ± 4.60                 | 107.23 ± 7.14              |
| Height percentile               | 39.54 ± 5.24                  | 34.64 ± 8.42               |
| Pain crises                     | 0.892 ± 0.31                  | 0.688 ± 0.24               |
| Units of blood                  | 1.333 ± 0.398                 | 0.563 ± 0.33               |
| Infections                      | 0.526 ± 0.15                  | 0.688 ± 0.18               |
| Hospitalizations                | 1.60 ± 0.35                   | 1.25 ± 0.43                |
| Hematocrit, %                   | 27.45 ± 0.81                  | 28.00 ± 1.08               |
| Hemoglobin, g/dL                | 8.81 ± 0.31                   | 8.673 ± 0.39               |
| White blood cells × 10⁶/mL       | 12.364 ± 0.79                 | 13.15 ± 1.23               |
| Health score d                  | 4.154 ± 0.77                  | 3.78 ± 0.72                |

*Inflammation is defined as C-reactive protein >10 mg/L and/or α1-acid glycoprotein >1.0 g/L.

P < 0.05.

P = 0.1.

Health score is the sum of the number of episodes of pain crisis, infection, hospital admission, and/or blood units received.

P > 0.1). IL-2 concentration in the supernatant of PHA-activated cultures was not significantly affected by inflammation (graph not shown). When cells were activated with 5, 10, and 15 μg/mL PHA, there was a small decrease in the mean IL-2 activity (13.3%-36.5%) with increasing health scores (Figure 3D).

Clinical Status and In Vitro Immunity in Children With β-Thalassemia

Despite the small sample size, data for selected measurements were also analyzed as a function of β-thalassemia subgroups. Children with HbSβthal genotype (n = 3) had a slightly higher mean (± SEM) CRP concentration (3.0 ± 1.70 mg/L) than children with HbSβ⁺thal genotype (n = 5) (0.62 ± 0.62 mg/L, P = 0.08). Mean (± SEM) plasma retinol concentrations were not significantly different between children with HbSβ⁺thal (53.53 ± 0.30 μg/dL) and those with HbSβ⁻thal genotype (56.60 ± 13.50 μg/dL). Also, no significant difference was observed between both groups with all other measurements (data not shown).

Correlation Coefficients and Multiple Regression Analysis

Lymphocyte proliferative responses to PHA positively and significantly correlated with retinol binding protein concentrations, negatively correlated with health scores, pain crises, and units of blood received (Table 6, P < 0.05), and nonsignificantly correlated with infection episodes. Although the same trend was observed between IL-2 concentrations in the supernatants of PHA-treated cultures and measures of clinical status, none of the correlations was statistically significant (data not shown). Plasma retinol concentrations positively correlated with weight percentile (r = 0.181), height percentile (r = 0.222), IL-2 concentration (5 μg/mL PHA only, r = 0.184), and number of blood units received (r = 0.329, P < 0.05). Plasma retinol concentrations negatively correlated with inflammation scores (r = 0.235, P < 0.05) and nonsignificantly with the number of pain crisis episodes (r = 0.170) and hospitalizations (r = 0.127). Multiple regression analysis with IL-2 and/or lymphocyte proliferative responses to PHA as dependent variables did not show any strong association with anthropometric measurements, inflammation, hematologic measurements, or sex.

DISCUSSION

Our study reveals a few important observations: (1) no child <5 years of age had a plasma retinol concentration below normal (<20 μg/dL); (2) vitamin A deficiency was observed in 7/59 children (nearly 12%), which suggests that vitamin A deficiency is significantly higher than that observed (0.6%-1.5%, 95% confidence interval, mean of 1%) in American children of the same age range;3,24; (3) the weight and height mean percentiles were <50th percentile, severe growth deficits (weight or height <3rd percentile) were not common in the overall population. Nearly 43% (3/7) of the children with plasma retinol below normal, compared with 22% of those with normal plasma retinol concentrations, had weight
below the 10th percentile. However, while no child with plasma retinol concentration below normal had height below the 10th percentile, nearly 7% of those with adequate plasma retinol levels did, and only 2 children in the overall population had height below the 3rd percentile. The lack of a high prevalence of severe growth deficits could be attributed to excellent healthcare and disease management and may also suggest that either macronutrient intake or utilization were adequate.

Although differences were small and not statistically significant, the overall health status (weight percentile, height percentile, and inflammation scores) appeared to be worse in boys than in girls. Boys also had lower mean plasma retinol levels and IL-2 activity in the supernatant of PHA-treated cells than girls. Although the experimental design did not allow us to identify the mechanisms of these differences, published data about adults without SCD have clearly demonstrated significant sex differences in the risk of infections and in immune responses to vaccines. Women have lower incidences and severity of many viral, bacterial, and protozoan infections than men, and they also mount stronger humoral and cell-mediated immune responses to infection. In a 2013 study, Sankaran-Walters et al reported that women had a higher degree of immune activation of CD4+ and CD8+ blood- and gut-associated lymphocytes than men, and women also produced higher mean Th1-associated cytokines (TNF-α, IL-1β, and IL-17) that are required to recover from respiratory infections but lower Th2-associated cytokines (IL-4 and IL-10). Sex differences that may increase the risk of certain types of viral and bacterial infections start in infancy or prepubertal periods, with higher incidences and mortality rates in boys than in girls 0-14 years of age. At least in adults, certain measurements of SCD severity vary by sex. In one study, the prevalence and severity of pain crises were reported to be lower in females than in males; and the mean age at death was higher in females (29.1 years) than in males (26.2 years). In our study, the mean number of pain crises did not vary by sex, and the mean number of infections was only slightly and nonsignificantly lower in girls than in boys. However, as expected, the mean concentrations of CRP, TNF-α, and inflammatory scores were higher in boys than in girls. These factors may explain in part the lower mean plasma retinol levels in boys than in girls (Table 4). Because of the paucity of data on differences in immune responses in boys vs girls with SCD or adult male and female patients with SCD, this is an area of interest for future studies.

We observed a positive and significant correlation between lymphocyte proliferative responses to PHA and retinol binding protein (a surrogate marker of vitamin A status, especially in the absence of inflammation or overt protein-energy malnutrition) but a negative correlation with health scores, blood units received, pain crises, and infections. Plasma retinol positively correlated with IL-2 concentrations in culture activated with 5 μg/mL PHA. From the current data, we are unable to state which came first: inadequate vitamin A status affecting health scores (pain crises, inflammation assessed by CRP) and in vitro immune function or the opposite. In either case, pain crisis episodes are usually associated with inflammation and reduced nutrient intake (including vitamin A), and these factors may in turn affect immune function.

Several studies have been conducted on vitamin A status in children with SCD and its influence on health status or the role of vitamin A status in lymphocyte proliferation and cytokine secretion in individuals and laboratory animals without SCD. The negative effect of suboptimal vitamin A status on immunity has been clearly demonstrated. In 2 studies conducted on blood lymphocytes obtained from healthy adult subjects without SCD, the addition of vitamin A (in the form of retinoic acid) to culture medium resulted in increased IL-2 secretion, lymphocyte proliferation, and dendritic cell maturation. However, to our knowledge,
Ours is the first study in which in vitro immune function (lym-
phocyte proliferation, IL-2 secretion) and plasma levels of
TNF-α were investigated as a function of vitamin A status
in children with SCD. The small differences persisted after
taking into account hemoglobin genotype and sex (compar-
ing boys with HbSS and VAD (n = 5) and boys with HbSS and VAA (n = 18); (C) in children with retinol binding protein (RBP) and plasma retinol below normal
(n = 6) and children with normal plasma retinol but RBP below normal (n = 31); and (D) as a function of health scores
(n = 29, n = 20, and n = 8 for health scores 0-2, 3-8, and >8, respectively). For 2A, 2B, and 2C, no significant differences in
mean stimulation indexes (SI) were found between children with plasma retinol levels <20 μg/dL and those with levels
≥20 μg/dL. For 2D, for each PHA concentration (2.5, 5, 10, or 15 μg/mL), a dose-dependent decrease in the rate of DNA syn-
thesis was seen with increased health scores (P < 0.05 to P = 0.1). Values are mean ± SEM. CPM, counts per minute; VAA, vitamin
A–adequate, VAD, vitamin A–deficient.

6/7 children with plasma retinol concentrations below nor-
mal were children with HbSS genotype, consistent with the
fact that patients with this hemoglobin genotype tend to
have a more severe disease than those with other geno-
types (for example, HbSC or HbSβthal).

We did not investigate the mechanisms of impaired lym-
phocyte proliferation in the current study. However, we can
cspeculate that these mechanisms could be related to differ-
ences in the number of immunocompetent T lymphocytes
between vitamin A–deficient and vitamin A–adequate chil-
dren or to impaired gene expression of IL-2, IL-2 receptors, or
other factors that regulate lymphocyte proliferative responses.
Low percentages of CD4+ and CD8+ T cells previously have been reported in patients without SCD but with vitamin A deficiency, and they were corrected after the addition of vitamin A supplements. Vitamin A, through its metabolite all-trans retinoic acid, has been shown to modulate adaptive immunity through various mechanisms such as regulation of T cell differentiation and activation, secretion of proinflammatory and antiinflammatory cytokines, expression of cell receptors, and proteins such as cyclin D3 that regulate T cell proliferation. Retinoic acid has also been shown to downregulate apoptosis of activated human lymphocytes in vitro and to regulate the expression of nuclear factors of activated human T cells that control the production and/or secretion of IL-2. Blocking of retinoic acid receptor alpha abrogated the retinoic acid effect on IL-2 secretion and cell proliferation. This finding implies that any of these mechanisms could be responsible for the reduced lymphocyte proliferation and/or IL-2 secretion in activated lymphocytes from children with SCD and below-normal plasma retinol concentration.

The observed concentrations of plasma retinol in our study were higher than those reported by other investigators who have studied American and non-American children with SCD. Three possible explanations could account for

Figure 3. Interleukin-2 (IL-2) concentrations in phytohemagglutinin (PHA)-treated and untreated peripheral blood mononuclear cells in children with sickle cell disease (A) as a function of plasma retinol levels (n = 52 children with normal plasma retinol levels and n = 7 children with deficient plasma retinol levels); (B) in boys with HbSS and plasma retinol below normal (n = 5) and boys with normal plasma retinol (n = 18); (C) in children with retinol binding protein (RBP) and plasma retinol below normal (n = 6) and children with normal plasma retinol but RBP below normal (n = 31); and (D) as a function of health scores (n = 29, n = 20, and n = 8 for health scores 0-2, 3-8, and >8, respectively). For 3A and 3C (PHA 0 μg/mL), the mean IL-2 concentrations of children with plasma retinol <20 μg/dL were higher than those with plasma retinol ≥20 mg/dL (a>b, P < 0.05 in 3A; †P = 0.052 in 3C). For PHA 5 and/or 15 μg/mL, the mean IL-2 concentrations of children with plasma retinol <20 μg/dL were lower than those with plasma retinol ≥20 μg/dL (*P < 0.1 but >0.05 in 3C). The means of IL-2 activity between children plasma retinol <20 μg/dL and those with levels ≥20 μg/dL (3B) and as a function of health scores (3D) were not statistically different. Values are mean ± SEM. VAA, vitamin A–adequate; VAD, vitamin A–deficient.
the difference: (1) methods used for plasma retinol assay, (2) use of vitamin supplements, and (3) differences in the population. In our study, plasma retinol was assayed by fluorometric methods, whereas other investigators have used high-performance liquid chromatography. Additionally, Dougherty et al included only children with HbSS genotype in their study.22 Because micronutrient (multivitamin-mineral) supplements are commonly used in the United States, this practice may, in part, explain the high plasma retinol concentrations in our studied population.

Our study has several limitations: (1) the small sample size, (2) cross-sectional nature of the study, (3) lack of information on vitamin A intake, and (4) lack of data on the mechanisms of impaired lymphocyte proliferation and/or IL-2 secretion. These issues will be addressed in a future study.

CONCLUSION

Our study suggests that approximately 12% of the researched children with SCD had plasma retinol concentrations below normal. The affected children had poorer in vitro immune function and health compared to those with an adequate vitamin A status. Considering the negative effects of poor immunity on infection, a predisposing factor for pain crisis, our data suggest that routine measurement of plasma retinol should be performed in children with SCD, especially those >5 years of age.

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