Intracellular hemoglobin S (HbS) polymerization is most likely to be the primary determinant of the clinical and biologic manifestations of sickle cell disease (SCD). Fetal hemoglobin (HbF) does not enter the HbS polymer and its intracellular expression in sickle erythrocytes inhibits polymerization. HbF levels, high at birth but decreasing thereafter, protect the newborn from the clinical manifestations of this hemoglobinopathy. We have measured the sequential changes in HbF, reticulocytes, and F cells in the first 2 years of life in 25 children with SCD and compared the results with those obtained in 30 normal children (AA). We have also calculated HbF per F cell (F/F cell), the preferential survival of F cells versus non-F cells, as measured by the ratio F cells versus F reticulocytes (FC/FR) and polymer tendency at 40% and 70% oxygen saturation. HbF levels decreased from about 80.4% ± 4.0% at birth to 9.2% ± 2.9% at 24 months. During this time, we observed a regular decrease of the F reticulocytes and the F cells. The kinetics of the decline of F/F cell was comparable with the decline of HbF, rapid from birth (mean, 27.0 ± 3.6 pg) to 12 months of age (mean, 8.5 ± 1.5 pg) and then slower from 12 to 24 months of age (mean, 6.2 ± 1.0 pg) in the SCD children. In the AA children, the decrease in HbF, due to changes in both numbers of F cells and F/F cell, was more precipitous, reaching steady-state levels by 10 months of age. Calculated values for mean polymer tendency in the F-cell population showed that polymerization should begin to occur at 40% oxygen saturation at about 3 months and increase progressively with age, whereas polymerization at 70% oxygen saturation would not occur until about 24 months. These values correspond to HbF levels of 50.8% ± 10.8% and 9.2% ± 2.9%, respectively, and F/F cell levels of 15.6 ± 4.5 pg and 6.2 ± 1.0 pg, respectively. In the non-F-cell population, polymerization was expected at birth at both oxygen saturation values. Three individuals had significantly greater predicted polymerization tendency than the remainder of the group because of early decreases in HbF. These individuals in particular, the remainder of the cohort, as well as other recruited newborns, will be studied prospectively to ascertain the relationship among hemoglobin parameters, which determine polymerization tendency and the various clinical manifestations of SCD.

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study is the first report of a long-term prospective analysis of the relationship between these parameters and various clinical manifestations in this cohort of children, and others of the relationship between these parameters and various studies. The Parisian Prospective Study on Sickle Cell Disease (PPSSCD).

MATERIALS AND METHODS

Patients. Twenty-five homozygous SCD children (SS) were selected for this study from the clinics of Necker-Enfants Malades Hospital and Robert Debré Hospital, Paris, France. Most of the children were identified through neonatal screening performed in our pediatric departments. These children were followed regularly in our clinic, offering the opportunity of longitudinal analyses. The age range for the data reported here was 3 weeks to 24 months. Each patient was studied every 4 months with a variable number of determinations according to the age of inclusion in the study. Blood samples were obtained from individuals who were in steady state (not experiencing a painful crisis or another acute medical condition) and with normal iron status. Diagnosis of SCD was established for each individual on the basis of hemoglobin electrophoresis and family studies.

Hematologic data. For each patient complete blood counts and erythrocyte indices, mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC), were determined using a Coulter Model STKR (Coulter Electronics, Hialeah, FL). Because of previous concern about the precision of electronic-based methodologies for determining MCHC, we conducted a pilot comparing study of MCHC measurements done manually or in this Coulter machine. We found an almost perfect agreement with a correlation coefficient of 0.928 (p < .001) for these two measurements. Reticulocyte counts were performed after methylene blue supravital stain.

Determination of a-globin genotype. The a-globin genotypes were determined by Southern blot or polymerase chain reaction (PCR) analysis according to the strategy previously described.16

Fetal hemoglobin production. HbF production was ascertained through measurements of the following parameters: HbF, F reticulocytes, F cells, F/F cell, and FC/FR. HbF was quantified by a high performance ion-exchange liquid chromatography (HPLC) procedure.19

Monoclonal anti-HbF and immunofluorescent staining of cells. Monoclonal antibodies against HbF were produced from splenocytes of BALB/c mice immunized with cord red blood cells (D. Blanchard, C. Willem, A. Gourbil, and M.J. Loirat, unpublished data). Briefly, splenocytes from a mouse immunized four times by intraperitoneal injections of 2 x 10^8 cells were fused with SP2/0-Ag14 myeloma cells according to conventional protocol.20 For hybridoma screening, culture supernatants were analyzed by enzyme-linked immunosorbent assay (ELISA) using crude hemolysates from cord and adult red blood cells and antigens.21 Hemoglobin preparations were coated at approximately 20 pg hemoglobin per mL in 0.1 mol/L carbonate buffer, pH 9.6. Antibodies that bound only to the crude hemoglobin extract from cord cells were selected, expanded, and further characterized by immunoblotting. The clone NaM16-2F4, obtained after two limiting dilutions,22 secreted an IgGl (kappa chain) which reacted with HbF, but not HbA on immunoblots. The antibodies were used as a standard for measurements of F reticulocytes and F cells.

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The cells were evaluated simultaneously by an original method derived from previously described techniques.22,23 Identification and quantification of these cells was performed on fixed blood smears following staining with thiazole orange and an indirect immunofluorescent assay using the monoclonal anti-HbF antibodies and a rabbit anti-immunoglobulin-fluorescein isothiocyanate (FITC). The procedure used 50 pL of whole blood mixed with an equal volume of 1% new methylene blue N. The mixed preparation was allowed to stand 10 minutes at room temperature to precipitate the reticulum in the reticulocytes. Films were prepared, air-dried, fixed 5 minutes at room temperature in acetone/methanol (9:1), and rinsed immediately in phosphate-buffered saline (PBS), pH 7.1 (NaCl, 8.50 g; Na2HPO4, 1.07 g; NaH2PO4, 0.39 g, made up to 1 L with distilled water). A working thiazole orange solution (100 pL) was layered over the films (the working solution was obtained by dilution (1:8) in PBS of a stock solution of 0.4 pmol/L thiazole orange prepared by adding 1 mg thiazole orange powder to 1 mL ethanol). The slides were incubated in a moist chamber for 30 minutes at 37°C. After rinsing in PBS, monoclonal antibodies anti-HbF (100 pL) were applied to the slides and incubated in a moist chamber for 30 minutes at 37°C. After rinsing in PBS, the slides were dried again and the rabbit anti-immunoglobulin-fluorescein isothiocyanate labeled (100 pL) was applied on the films, and incubated in a moist chamber for 30 minutes at 37°C. After a rinse in PBS, the slides were dried and examined with a fluorescence microscope equipped with the appropriate filters for FITC. With this method, four types of cells were stained: reticulocytes with HbF (F reticulocytes) showed the orange reticulum and the green fluorescence, reticulocytes without HbF (non-F reticulocytes) showed the orange reticulum and no green fluorescence, erythrocytes with HbF (F cells) were green and erythrocytes without HbF (non-F cells) were not fluorescent and appeared brown. The number of fluorescent cells were counted after switching to the UV light. Several counts (3 x 100 cells) were necessary to quantitate the percentage of F reticulocytes versus the total reticulocytes. The same procedure was used to measure the percentage of F cells versus the total erythrocytes.

F/F cell was calculated using the following ratio: (MCH x HbF) / F cells.

FC/FR was the enrichment ratio of F cells versus F reticulocytes.

The changes of the different parameters HbF, F cells, and FC/FR cell as a function of age in SS patients were compared with those obtained in normal children (AA) studied by the same method.

Polymer calculations. The values of predicted levels of intracellular HbS polymer fraction have been calculated at 40% and 70% oxygen saturation values, which correspond to the physiologically relevant region of oxygen saturation, from the algorithms previously described.17 The calculations are based on the two-phase model with the polymer phase at a concentration of 69 g/dL (Cp). Polymer fraction is equal to Cp (MCHC-Cs)/(MCHC(Cp - Cs)) where Cs, the solubility of the intracellular hemoglobin at any oxygen saturation, is calculated from data on hemoglobin solutions,24 Polymer is calculated separately for F cells and non-F cells at the average cell density (MCHC).

Statistical analysis. Statistical analyses were done using the t-test for variables with Gaussian distribution and SAS 6.07 Software (Cary, NC). A mixed analysis variance using PROC MIXED24 was performed to compare the slopes of HbF, F cells, and FC/FR cell decreases with age, in SS patients and AA children. A logarithmic transformation of the biologic data was first done to linearize the relationships between the age and the parameters.

RESULTS

Hematologic data. The determination of the total hemoglobin (g/dL) showed a value of 10.8 ± 0.9 g/dL at 1 month, which decreased to the significantly lower value of 9.0 ± 0.2 g/dL by 2 months, with the gradual fall after this period until a value of 7.3 ± 0.5 g/dL by 24 months. MCHC levels (g/dL) averaged at birth 33.6 ± 0.7 g/dL and at 24 months 32.9 ± 1.6 g/dL (Table 1). These results are similar to those reported by Serjeant.25 The reticulocyte count was increased early in the life: high values were observed as soon as the
second month of life (252 ± 46, 10^9/L), suggesting significant levels of hemolysis with important interindividual variations (data not shown).

**HbF production.** Changes in levels of F reticulocytes (%) were studied and showed a steady decrease from 66.7% ± 19.7% at 1 month to 13.4% ± 4.5% at 24 months, with the major change occurring in the first 6 months of life (Fig 1). The variations of HbF levels (%), F cells (%), and F/F cell (pg/cell) are represented in Fig 2. The same determinations were made in 30 AA children and the results are also reported in Fig 2. In SS children, HbF levels declined rapidly from the first month (80.4% ± 4.0%) to 8 to 10 months (28.2% ± 4.5%) and then more slowly after this age (24 months = 9.2% ± 2.9%). The percentage of F cells decreased at a quite steady rate from the first month (88.7% ± 3.3%) to 24 months (43.4% ± 1.5%). The F/F cell fell most rapidly from the first month (27.0 ± 3.6 pg) to about six months; reaching 8.7 ± 1.0 pg by 12 months with a further decrease to 6.2 ± 1.0 pg by 24 months. Thus, much of the initial decrease in HbF levels is due to the decline in F/F cell, whereas after the first year, the change in number of F cells becomes predominant. As previously described, the decrease of HbF in SS children, was delayed when compared with that in AA children. We found the same phenomenon for the decreases of F cells and F/F cell (Fig 2 and Table 2). The statistical comparisons of the declines in both populations are highly significant for all parameters (P < 10^-4). The values of the ratio FC/FR in SS children were always higher than 2 and showed great variations (data not shown).

**Polymer formation at different oxygen saturations.** Polymer tendency was calculated for two values of oxygen saturation of relevance physiologically, 40% and 70%, from data on MCHC and average F/F cell for both F cells and non-F cells.

In the F-cell population (Fig 3), at 40% O2 saturation, no polymer would be expected during the first 3 months except for one child (patient 1) of the 25 children studied during this period. Polymerization would be expected to occur in an average density cell at this oxygen saturation at about 3 months of age and to rise progressively during the first 24 months. Interestingly, three patients would be expected to have strikingly elevated polymer fraction as compared with others at that age (patient 1 was studied at 1 and 6 months, patient 2 at 14 and 24 months, and patient 3 at 18 months). At 70% O2 saturation, no polymerization would be expected in any of the F-cell populations before 2 years of age, except for the patients 2 and 3.

In the non-F-cell population (Fig 3), at 40% O2 saturation, significant polymerization would be expected even at birth and, as predicted from the lack of major changes in MCHC, would be expected to remain relatively constant until 24 months. At 70% O2 saturation, traces of polymer fraction would be expected at birth and again would show minimal variations from birth to the age of 24 months.

**Concomitant variations of F/F cell and polymerization process.** Figure 4 shows the decrease of F/F cell in F-cell population and the progressive increase of the expected polymer fraction of HbS at 40% O2 saturation as a function of age. Figure 5 represents expected formation of polymer in F cells at 40% O2 saturation as a function of F/F cell. Polymer would not be expected to appear above the threshold of F/F cell of 15 pg. At lower values of F/F cell, the polymerization process was inversely correlated to the level of F/F cell.

**Effects of α-thalassemia and gender on HbF production and the polymerization process.** We examined the effects of α-thalassemia on the variations of F/F cell and the expected polymer fraction of HbS at 40% O2 saturation by comparison of the values of these two parameters obtained.
in children with three \((n = 7)\) and four \((n = 9)\) \(\alpha\) genes studied at the same age. We found no significant differences between male \((n = 14)\) and female \((n = 11)\) patients with or without \(\alpha\) thalassemia (data not shown) in these parameters; but this lack of difference is perhaps due to the small number of patients in each group.

**DISCUSSION**

Strong arguments support the hypothesis that intracellular polymerization of HbS is the primary determinant of the severity of SCD in different populations with the various sickle cell syndromes.\(^6\) Polymerization depends, for any erythrocyte population, primarily on oxygen saturation, intracellular hemoglobin concentration, and hemoglobin composition.\(^7,10\) Non-S hemoglobins, such as hemoglobins F, A, A\(_2\) decrease the extent of polymerization by decreasing the amount of HbS and also by the formation of mixed hybrids with HbS with reduced or absent tendency to enter the polymer.\(^12,21\) The objectives of our study were both to analyze changes with age of the cellular parameters resulting in the decline of HbF levels after birth, and at the same time estimate the expected appearance of significant intracellular polymer in a population of SCD infants before two years of age.\(^23\) Estimations of the sparing effects of non-S hemoglobins on the polymerization of deoxygenated HbS must take also into account that the distribution of HbF within the erythrocytes...
is heterogeneous. The calculation of polymer fractions was thus done in this work considering first the F-cell population and secondly the non-F-cell population. When using the parameter F/F cell instead of the total HbF in the calculation of polymers in the F-cell population, the reduction in polymer formation was greater than would be predicted from the total HbF. In the non-F cells, which contain no detectable amounts of HbF, there will be no change in polymer formation, except as reflected in heterogeneity in intracellular hemoglobin concentration.

The effect of changes in MCHC on polymerization tendency is much smaller than the changes in HbF parameters, despite the great sensitivity of polymerization to MCHC, because we find, as was reported by Serjeant, that the fluctuations in this parameter are relatively small during this period of observation of the cohort.

While considering separately the different variables regulating the levels of HbF, we observed a regular decrease of the F reticulocytes and the F cells, but the decline of F/F cell was most rapid from birth to 12 months, and then slower from 12 to 24 months. The kinetics of the decline of F/F cell was comparable to the kinetics of the decrease of HbF. It can be thus postulated that the diminution of HbF before one year of age, is mainly related to the diminution of the parameter F/F cell. In AA children, the decrease of HbF was much more precipitous than in SS children and the main
When examining hematologic data of the cohort, we observed that, as previously described, total hemoglobin fell rapidly from birth to the age of 2 months, whereas reticulocyte counts increased to a mean of 252 ± 46, 10^6/L. The high values of the ratio FC/FR prove the existence of a selection of F cells versus non-F cells and give a pathophysiologic basis to the observation already made by Serjeant. Early polymerization in non-F cells most likely explains the possible occurrence of clinical manifestations of the disease in the first months of life.

At least, three children in this study were of a particular interest because they differed markedly from the rest of the population, exhibiting a high expected polymer formation at early age, due to a particularly low Hbf production. In patient 1, the decrease of F/F cell occurred early despite a high level of Hbf and F cell levels comparable to those observed in children of the same age. In patient 2, the rapid decrease of Hbf was related to a precocious decrease of F cells, while F/F cell did not differ from those of age-matched patients. The mechanism of the rapid decline of Hbf in patient 3 was linked to the decrease of both F cells and F/F cell. These facts illustrate the different cellular parameters likely to influence the polymerization process and the need to consider F/F cell and the proportion of F cells versus non-F cells to understand the pathophysiology of the sickling phenomenon. These three cases showed that both variations of F/F cell and F cells are independent and possibly regulated by different genetic factors. It is obvious that the clinical follow-up of these three patients, compared with the evolution of the rest of the cohort, will be of a particular interest. Our hypothesis is that low Hbf levels and resultant high-polymer tendency will be followed by the occurrence of more frequent complications, at early ages, of SCD in these babies.

It is our intention to follow closely these patients, and the remainder of the 25 individuals who have entered the study, as well as continuously, recruit newborn sickle cell infants in Paris into the study over the next several years. Hematologic and clinical parameters will be serially ascertained and correlated. As our data accumulate, we will be able to determine whether a gene status, gender, or haplotype influence these parameters and clinical correlations. If such correlations are observed, studies of cellular factors influencing Hbf expression and polymer formation, would offer early prognostic indices, which are highly needed in SCD to guide the application of new and experimental therapies.


Variation in fetal hemoglobin parameters and predicted hemoglobin S polymerization in sickle cell children in the first two years of life: Parisian Prospective Study on Sickle Cell Disease

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