Dense Cells in Sickle Cell Anemia: The Effects of Gene Interaction

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In an attempt to uncover potential genetic sources of the clinical diversity of sickle cell anemia, we have characterized homozygous SS patients in the following ways: percentage of dense red blood cells (% F4) as determined from Percoll-Stractan continuous density gradients, alpha gene deletion, average percentage of hemoglobin F (% HbF), hemoglobin in g/dL, age, and sex. We find that alpha 4 individuals have a higher % F4 (mean 24% ± 15%) than alpha 3 individuals (mean 12% ± 8%) (P < .006). Multivariate analysis demonstrated a significant correlation among % F4 levels and α-gene number and % HbF, and an interaction between the last two variables. The other variables considered did not significantly alter this model. As reported before, with fewer samples, we find that in the first ten years of life of SS individuals, the frequency of alpha gene deletion is 17%, which is comparable to that in the general black population, while in the group over 20 years of age, the frequency rises to 48%, implying that alpha thalassemia is associated with longer survival. These results indicate that it is necessary to consider sickle cell anemia not only as a single gene defect, but also as a disease whose clinical expression is the result of a group of genes capable of interacting at the phenotypic level.

SICKLE CELL ANEMIA is a genetic disease with a variable clinical expression. Most patients have a course of moderate severity, but both severe and mild courses are also seen. Some of this variation may arise from environmental conditions: it has been shown that cold can induce dactylitis; dehydration induced by hot weather has been implicated in the generation of sickle cell painful crisis; and concomitant endemic infections, like malaria, can alter the clinical course of sickle cell anemia. Nevertheless, when patients living in similar surroundings and exposed to the same climate are studied, it becomes apparent that interaction with other physiologic characteristics (percent hemoglobin F, alpha thalassemia [alpha 2 or alpha 3], microcirculatory structure), some of which may be under genetic control, have to be postulated to explain most of the differences in clinical expression.

Our laboratory questioned whether or not the red cell density heterogeneity among sickle cell anemia patients could be correlated with the presence or absence of other genes or features of the disease likely to be under genetic control. We have previously demonstrated that the distribution of red cell density varies considerably from one patient to another and is stable in individual patients over periods of at least 14 months in the absence of crisis. In addition, the populations of cells harvested from different density levels contain cells with distinct rheologic and hemodynamic properties. This article deals with establishing some of the genetic origins of this variance in an effort to understand the larger question of the interaction of the beta S gene with other genes in the generation of the clinical picture of sickle cell anemia.

MATERIALS AND METHODS

Patients

One hundred patients with sickle cell anemia who are being followed in the hematology clinics associated with the Division of Hematology at the Albert Einstein College of Medicine, Bronx, NY, and at Lincoln Hospital, Bronx, were included in this study. To identify homozygous SS patients, two electrophoreses were performed, one at pH 8.6 (cellulose acetate) and another at pH 6.4 (agar), accompanied by a solubility test in high phosphate buffer. Efforts were made to eliminate S/α thalassemia by pedigree analysis and red cell indices, but some of these patients might have been included in the sample. Patients who had been treated by transfusion during the preceding three months were excluded from the study.

Density Gradients and Their Evaluation

Percoll (colloidal silica coated with polyvinylpyrrolidone; Pharmacia, Inc, Piscataway, NJ) and Stractan (arabino-galactan polysaccharide; St Regis Paper Co, West Nyack, NY; prepared as described by Corash et al) gradients were formed from a mixture of Percoll-Stractan (density 1.207, determined from the refractive index): water:10x balanced salts = 3.5:3.0:2.8:0.7. The initial pH and osmolarity were adjusted to 7.35 and 290 mosm, respectively. The mixture was made in batches large enough to make 200 to 300 determinations and was frozen in 50-ML portions. To determine the red cell density distribution, a 0.1-ML aliquot of a well-mixed sample of whole blood, with the hematocrit adjusted to 50 and preserved in citrate-phosphate-dextrose (CPD), was added to 5.9 mL of the gradient mix; the tubes were mixed by inversion; and finally they were spun in a Sorvall 55-34 rotor for 40 minutes at 17,000 rpm (35,000 g) at 38 °C. Each group of tubes included blood from a known AA individual and a tube with Pharmacia density marker beads to insure that conditions remained constant from one centrifugation to the next. The resulting gradient is continuous but nonlinear.

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The formed gradients were photographed with Polaroid 46-L continuous density transparency film to form an image of the gradient 5.2 cm from the meniscus to the bottom of the tube and were read in a Corning model 720 densitometer. Chartpak tape with 12 divisions to 5.2 cm was used to mark 12 equal divisions from the meniscus to the bottom of the tube, which were then read in the manual mode of the Corning densitometer, which ignores the first and last divisions and results in ten density readings. The % F4 is the sum of the last three divisions, as indicated in Fig 3. The lightest cells in F4 have a mean corpuscular hemoglobin concentration (MCHC) of approximately 40 g/dL. The % F2 is the sum of divisions three to five. The validity of this process can be demonstrated by comparing the results from the densitometer method to those obtained by a manual method, which consists of physically separating the cells into four density fractions; adjusting the total volume of the washed fraction to a constant volume (in this case 1 ml), and determining the hemoglobin content of each fraction. For one individual who was extensively studied by both methods, the mean of 13 manual determinations of fraction 4 gave a value of 18% ± 3%,* while the mean of three measurements by densitometry gave a percentage for fraction 4 of 16% ± 2%.

Alpha Gene Determination

DNA was prepared from the subjects' peripheral blood, and 3-µg aliquots were digested to completion with Bam H1 (New England Biolabs, Beverly, Mass) in the buffer recommended by the manufacturer. Following 0.9% agarose electrophoresis, the DNA fragments were transferred to nitrocellulose filters (Schleicher and Schuell, Keene, NH) by Southern’s method. Plasmid JW 101 (alpha globin cDNA) was nick-translated in the presence of 32P nick-translated probe (32P-NTPb) to yield a specific activity of 106 cpm/µg DNA.

Following filter hybridization at 65 °C for 24 hours, the blots were analyzed by autoradiography. DNA samples with 14 kilobase (kb) bands only were scored as alpha 4 (alpha/alpha); those with 10.5-kb bands only were scored as alpha 2 (-alpha/-alpha); and those with both 10.5- and 14-kb bands were scored as alpha 3 (alpha/alpha). Theoretically, alpha/-alpha could be confused with alpha 4, but this genotype is very rare in the black population.

HbF Determination

The alkaline denaturation method, as described by Betke et al., was used.

Statistics

The data were analyzed using the Apple II by standard multivariate methods.

RESULTS

The total sample consisted of 100 patients who were homozygous for hemoglobin S. Patients with known S/beta thalassemia, as determined by analysis of family members, were excluded from the study; however, some undetected S/beta thalassemic individuals may be included. Complete information was available for 43 of these patients over 10 years of age for whom density gradient profile, hemoglobin, percent hemoglobin F, alpha gene determination, sex, and age were obtained. This sample contained approximately equal numbers of males (N = 20) and females (W = 23). The alpha gene distribution was alpha/alpha (alpha 4) (N = 22), alpha/-alpha (alpha 3) (N = 18), and -alpha/-alpha (alpha 2) (N = 3). The mean age of the alpha 2 sample was 26.3 years, the mean age of the alpha 3 sample was 28.6, and the mean age of the alpha 4 sample was 24.0. Figure 1 shows density gradient patterns for six representative individuals. A histogram showing the number of individuals with a given percentage of fraction 4 is shown in Fig 2. The age, sex, and alpha gene distribution of these 43 patients is not significantly different from the 73 out of 100 individuals over age 10 shown in Fig 5.

HbSS patients classified into alpha/alpha and alpha/-alpha genotypes exhibited two distinctive density gradient patterns (Fig 3) when the mean of the percentage of cells found in each of ten equal divisions of the gradient was calculated as described in Materials and Methods. The most striking difference was the decrease in the very dense fraction of cells in the alpha 3 patients. This is the fraction that contains between 40% and 70% irreversibly sickled cells (ISC). Patients with four alpha genes had a clearly bimodal distribution, with a mean of 24% ± 15% of their cells in the densest fraction (F4) and 44% ± 18% of their cells in the
Fig 3. Plot of the mean percent red cells in each of ten density classes determined from densitometer readings for 22 alpha 4 (αα/αα) and 18 alpha 3 (αα/−α) SS patients. Density class 1 is the lightest class and class 10 is the heaviest class. The density classes were combined as indicated to arrive at percent cells in fraction 1 (F1) through fraction 4 (F4). Note that the average alpha 4 individual has a clearly bimodal density distribution, while the average alpha 3 individual has very few dense cells. The error bars indicate the standard error of the mean.

Fig 4. The percent of fraction 4 for alpha 3 (αα/−α) and alpha 4 individuals (αα/αα). The heavy bar is the mean; the dotted bars are 1 SD. Equality of means, $P < .005$; equality of variance, $P < .02$.

The effect of an interaction between α-gene status and HbF is apparent in that in non–α-thalassemic patients, a strong inverse correlation exists between log % HbF and fraction 4, while in alpha 3 patients, there is no significant relation. The $F$ statistic for this model is $F_{3,36} = 9.310$, which corresponds to a $P$ value of .001.

We have correlated age with the percentage of cells in fraction 4 for the sample of 43 patients, plus four
additional patients over 40 who did not have alpha gene analysis. When the sample is divided between individuals older than 40 and younger than 40 years of age, the mean percentage F4 for those under 40 years of age was 20.4% ± 13.9%, while for those over 40 years, it was 9.5% ± 4.31%. The difference in these means is significant at P > .05.

We have also correlated the frequency of alpha gene deletion with age in a sample of 100 homozygous SS patients ranging in age from birth to 61 years. We find that between 0 and 10 years, alpha 2 and alpha 3 individuals represent 17% of the population; between 10 and 20 years of age, the percent of alpha 2 and alpha 3 individuals rises to 38%; and for the individuals over 20 years of age, the percentage rises to 49% (Fig 6).

**FIGURE 6.** Histogram showing the relative number of patients with −α/ −α, αα/ −α, and αα/αα as a function of patient age. The frequency of αα/ −α and −α/ −α in the 0 to 10 year group is 17%; the frequency in the 10 to 20 group is 38%, and the frequency in the over 20 group is 49%.

**DISCUSSION**

The data presented here demonstrate that the heterogeneity observed in the density distribution of red cells in sickle cell anemia patients is the consequence, at least in part, of the action of other genes on the phenotypic expression of the homozygote state for hemoglobin S.

As seen in Figs 3 and 4, when the alpha thalassemia genotype (αα/ −α) coexists with homozygous HbS, a significant reduction in the number of very dense cells is observed. This accounts for the very asymmetric distribution of the percent dense cells in the patient population over 10 years of age seen in Fig 2. The large population of patients with very few dense cells consists predominantly of alpha 2 or alpha 3 individuals, while the second broader distribution of patients with more dense cells consists mainly of individuals with all four alpha genes. It has been previously reported that elevated levels of Hbf are associated with decreased numbers of ISCs.13 We find here that there is a very strong inverse correlation between the % F4 and the % Hbf for patients who do not have alpha thalassemia (Fig 5), but that there is very little effect of the % Hbf on the % F4 for αα/ −α individuals. A model developed by multivariant analysis that takes into account alpha gene deletion and % Hbf accounts for 46% of the variance. Therefore, other factors (genetic and non-genetic) might be operating and should be the objective of active search in the future.

It is interesting to examine the possible mechanisms by which alpha 3 and Hbf reduce the percentage of dense cells. Alpha thalassemia probably reduces the concentration of hemoglobin in the red cells of these patients, an alteration that is accompanied by a diminution in the propensity to sickle due to the exceedingly high dependence of polymerization on initial hemoglobin concentration.14 Evidence for this physiopathologic explanation comes from the demonstration of reduced MCHC in these patients.15,16 The case for Hbf interference in the polymerization of Hbs is quite strong. The initial in vitro demonstration that Hbf inhibits the polymerization of Hbs17 has been followed by the demonstration that hybrid formation (of the type alpha 2/beta gamma) is indispensable for the inhibitory effect of Hbf to be expressed.18 Moreover, the actual residues in the surface of the gamma chain involved in the inhibition have been identified.19 Hemoglobin F has been shown to inhibit sickling,20 and the extent of K⁺ leak has been recently associated with the intracellular level of Hbf.21 This finding connects the Hbf level, and its implicit effect on the polymerization of Hbs, with the main mechanism that increases cell density22: the deoxygenation-mediated K⁺ efflux. We can conclude from this that Hbf decreases the production of dense cells because it inhibits the intraerythrocytic polymerization of Hbs.

The lack of relationship between dense cells of SS patients with alpha 3 and Hbf level is not unequivocally interpretable. It could be that the decrease in MCHC and the inhibitory effect of Hbf are not additive or that the preservation of splenic function for a longer period of time in alpha 3 patients makes the removal of dense cells in low Hbf individuals more efficient.

The relationship between dense cells and age is more complex. We find that the number of dense cells decreases significantly in individuals with sickle cell anemia over the age of 40. There are three possible explanations for this finding: (1) individuals with a low percentage of dense cells live longer; (2) a small number of dense cells is associated with another factor associated with survival; or (3) dense cells decrease with age in all SS individuals. Only a prospective study will distinguish between these three possibilities. However, the correlation between reduced fraction 4 and alpha 3 demonstrated here, and the increased survival of alpha 3 individuals demonstrated by Mears et al10 and further amplified here, support the second possibility. In our larger study, we find that in the 0- to
10-year age group, the frequency of alpha gene deletion (alpha 2 or alpha 3) is 17%, which is comparable to that in the general black population in our area. From 10 to 20 years of age, the percent of alpha gene deletion rises to 38% of the SS population, while in the group over 20 years of age, the frequency rises to 49% (Fig 6).

To understand the increased survival of sickle cell anemia patients with alpha thalassemia, future research will have to investigate whether or not the increased incidence of splenic sequestration among these patients is significantly offset by a decrease in production and increase in removal of defective cells, as the splenic function might be retained for longer periods of time.

In conclusion, about half of the variance in cell density distribution found among sickle cell anemia patients can be accounted for by the presence of alpha 3 or the level of fetal hemoglobin. Further investigation will be needed to determine the origin of the remaining variance.

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ADDENDUM

After this manuscript was submitted, a paper by Embury, Clarke, Monrey, and Mohandas appeared that independently confirms the inverse correlation between dense cells and alpha thalassemia.

REFERENCES

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