Mutations responsible for hereditary spherocytosis (HS) lie in a variety of genes encoding transmembrane proteins (i.e., band 3), membrane skeletal proteins (i.e., β- and α-spectrin) and proteins mediating the attachment of the latter to the former (i.e., protein 4.2 and ankyrin). Analysis of erythrocyte membrane proteins by electrophoresis on polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS-PAGE) remains an unmatched element of orientation toward the primarily mutated protein (and gene). Human erythrocyte ankyrin is a mixture of different size proteins. The major form (protein 2.1) is a 206-kd protein. Other faster migrating ankyrin species, designated proteins 2.2 (186-kd), 2.3 (170-kd) and 2.6 (145-kd) appear as faint bands on stained gels. Band 2.3 and 2.6 are thought to stem from band 2.1 through proteolysis. The conversion is incomplete in reticulocytes. Protein 2.2 arises from alternative splicing and its concentration is unchanged during erythrocyte aging. Protein 2.1 is the only ankyrin isoform commonly evaluated with SDS-PAGE in HS patients. To estimate the extent of the distortion of band 2.1 and its derivatives due to high reticulocyte count we evaluated protein 2.1 levels in 10 unrelated HS patients with mutations in the ANK1 gene inactivating one ankyrin allele. A simple and practical equation was then worked out for obtaining the real ankyrin level.

Materials and Methods

Ten unrelated patients with typical HS were studied. Expression of ankyrin alleles was evaluated, as previously described, taking advantage of the (AC)n microsatellite polymorphism placed in the 3’ untranslated region of ankyrin DNA. Briefly, comparing DNAs and cDNAs relative to this region, we demonstrated the absence of the mRNA produced by one ANK1 allele. Two venous blood samples were drawn at different times, i.e., before splenectomy and at least six months after splenectomy. Erythrocyte ghosts were prepared and membrane protein concentration assessed as previously reported. SDS-PAGE was performed utilizing the continuous buffer system of Fairbanks with exponential gradient of acrylamide from 3.5% to 17%.

Results

Molecular biology, showing the lack of one ANK1 allele expression, clearly demonstrated that the primary alteration producing HS in these patients concerned the ANK1 gene. Despite molecular evidence, we found normal or greater than normal protein 2.1 levels in the erythrocyte membrane of these patients (mean ankyrin content ± SD, compared to normal controls: 110±15%). All patients had increased reticulocyte counts [mean reticulocyte count (10^9/L) ± SD: 380±90]. The erythrocyte membrane from the same patients, once splenectomized, showed a homogeneous degree of protein 2.1 reduction. Thus protein 2.1 levels could misleadingly appear normal due to the high number of circulating reticulocytes.

To calculate the true ankyrin level using PAGE and consequently to avoid mistakes in studying a mutated gene, a simple equation, based on the number of reticulocytes, was developed.

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splenectomized, showed, in agreement with the molecular results, a homogeneous degree of protein 2.1 reduction (mean ankyrin content ±SD, compared to normal controls: 78±8%) (Figure 1). Reticulocyte counts fell to normal levels (mean reticulocyte count (10^9/L) ±SD: 50±15). We evaluated the difference between the ankyrin levels found before and after splenectomy for each patient. These values and the reticulocyte counts before splenectomy were plotted and a significant, direct correlation was found (R-squared: 0.80) (Figure 2). Therefore, the higher the reticulocyte count, the more the ankyrin level appeared to be increased. The following simple equation was devised to normalize the ankyrin content obtained by SDS-PAGE on the number of circulating reticulocytes:

\[
\text{percentage of protein 2.1 to subtract} = 0.095 \times \text{number of reticulocytes (10}^9/\text{L}) + 8.744
\]

**Discussion**

As established by studies of spectrin and ankyrin synthesis in erythroblasts, spectrin deficiency is usually secondary to a primary reduction of ankyrin. Therefore, when a combined spectrin and ankyrin deficiency is detected, ANK1 gene should be investigated whereas α- or β-spectrin genes are generally implicated in HS with isolated spectrin reduction. American and European groups, studying by SDS-PAGE membrane proteins in HS patients, erroneously established that isolated spectrin deficiency was the most common biochemical alteration. In contrast, when measuring erythrocyte spectrin and ankyrin by radio immunoassay, Savvides et al. concluded that most HS patients have combined ankyrin and spectrin deficiency. Molecular biology, agreeing with this data, has recently and conclusively demonstrated that ankyrin mutations are a major cause of dominant and recessive HS.

In the great majority of laboratories, SDS-PAGE is the method of choice to study red cell membranes. However, since a high number of reticulocytes can mask the real ankyrin deficiency, the above equation should be used to avoid mistakes in approaching the mutate gene.

**References**