The Range of Hemoglobin A_2 in Hemoglobin E Heterozygotes as Determined by Capillary Electrophoresis

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Key Words: Hemoglobin A_2; Hemoglobin E; Capillary electrophoresis; Hemoglobinopathy; Heterozygote

DOI: 10.1309/AJCPP50JIXXZVLSS

Abstract

Capillary electrophoresis (CE) is capable of distinguishing hemoglobin E (HbE) from hemoglobin A_2 (HbA_2), thus permitting quantification of HbA_2 in patients with HbE. In this study, routine samples submitted for evaluation of hemoglobinopathy that demonstrated HbE were studied by high-pressure liquid chromatography and CE. The data for 52 samples from adult HbE heterozygotes were compared with those for a control group consisting of 209 patients. The mean HbA_2 of patients with HbE trait was 3.4% (SD, 0.4%), which was significantly higher ($P < .001$) than the 2.6% (SD, 0.4%) for the control group. Seven samples from adults homozygous for HbE were also evaluated. The mean HbA_2 of HbE homozygotes was 4.4%, which was significantly greater ($P < .001$) than the HbA_2 values for the HbE heterozygotes. Data from these cases provide an estimate of the range of HbA_2 in patients with HbE when evaluated by CE.

Hemoglobin E (HbE) is a variant that is second only to hemoglobin S (HbS) in prevalence.[1-5] It is hypothesized that the prevalence of HbE results from protection of RBCs from invasion by *Plasmodium falciparum*.[6-8] The highest frequency of HbE is found in India (especially its northeastern states) and Southeast Asia (especially southern Laos, eastern Thailand, and northeastern Cambodia).[4,9-12] It is thought that HbE-β-thalassemia is the most common form of thalassemia major in many parts of Asia.[13]

The *HbE* gene is a mutant form of the β-globin (*HBB*) gene that encodes lysine instead of glutamate at position 26. This β-E chain is inefficiently produced because of a novel cryptic messenger RNA splice site, leading to thalassemic RBC indices.[14] Furthermore, HbE has somewhat enhanced sensitivity to oxidant stress.[5]

Inheritance of HbE results in a spectrum of clinical phenotypes, depending on dosage, coinheritance of other hemoglobin variants, and environmental modifiers.[15] Heterozygosity (HbE trait) and homozygosity (HbEE disease) are clinically mild, whereas compound heterozygosity for HbE and HbS (HbSE) and compound heterozygosity for HbE and β-thalassemia (HbE-β-thalassemia) are clinically severe.[4,5,13,16,17] The identification of patients with heterozygous β-thalassemia is aided by the presence of increased levels of hemoglobin A_2 (HbA_2) together with a thalassemic hemogram. Thus, the capacity to measure HbA_2 in the presence of HbE by means of a rapid and widely available method is desirable.

In routine alkaline electrophoresis, HbE migrates with C, O Arab, and A_2. In acid electrophoresis, it migrates with HbA_2. High-pressure liquid chromatography (HPLC) is a rapid and automated method that has proven capable of identifying most hemoglobin variants while providing precise

measurement of HbA_2 and HbF in a range of hemoglobin genotypes[18-22]; however, the currently approved HPLC methods cannot separate HbE from HbA_2. While reverse phase HPLC has been reported to provide an estimate of HbA_2 in the presence of HbE, this method is not routinely used in clinical laboratories.[23] Thus, it has traditionally been impossible to precisely determine the quantity of HbA_2 in the presence of HbE, and definitive diagnosis of concomitant thalassemia required DNA testing.[4,15]

Recently, however, the Sebia Capillarys capillary electrophoresis (CE) system (Sebia, Norcross, GA) and the Tosoh HPLC analyzer (Tosoh Bioscience, South San Francisco, CA) have been reported as capable of distinguishing HbE from HbA_2.[24-27] CE is a method that has been effective for evaluation of serum proteins[28-33] and has recently been applied to the detection of hemoglobin variants.[33-36] CE has been demonstrated to be comparable to HPLC in the detection of hemoglobin variants; furthermore, it has proven superior to HPLC in the measurement of HbA_2 in the presence of HbE.[24,35,37] The present study was undertaken to determine the range of HbA_2 in patients with HbE.

Materials and Methods

Routine samples submitted to our reference laboratory for evaluation of hemoglobinopathy that demonstrated a phenotype consistent with heterozygous or homozygous HbE in our screening HPLC were identified. Samples obtained from children younger than 1 year were excluded. A total of 65 samples were obtained. These were compared with the previously published mean of a control group consisting of 209 patients with no hemoglobinopathy demonstrable by CE or HPLC.[24] Our study was conducted in accordance with a protocol (R-06-703) approved by the institutional review board of St Joseph Mercy Hospital, Ann Arbor, MI.

HPLC was performed using the Primus Resolution HPLC method (Primus, Kansas City, MO) as described previously.[19,24] This method relates the retention time of unknown hemoglobins to that of a calibrating standard containing 4 hemoglobins: HbF, HbA, HbS, and HbC. Whole blood specimens collected in EDTA were lysed with the hemolyzing reagent (provided by the manufacturer) for injection into the HPLC column. Elution of adsorbed hemoglobins used a gradient formed by 2 mobile phases of bis-tris (hydroxymethyl)aminomethane and 1 mmol of potassium cyanide with different pH values and ionic strengths, as previously described.[19,21]

CE was performed using the Sebia Capillarys system, as described previously.[24] Electrophoresis was performed in alkaline buffer, pH 9.4, provided by the manufacturer (Sebia). The hemoglobins were measured at a wavelength of 415 nm.

The presence of doubly heterozygous HbE-β-thalassemia was inferred from the combination of thalassemic hemogram parameters and percentage of HbE more than 3 SD from the mean for patients with the HbE trait. Similarly, the presence of doubly heterozygous HbE-α-thalassemia was inferred from the combination of thalassemic hemogram parameters and a percentage of HbE less than 3 SD from the mean for the patients with HbE trait. Genetic studies were not performed on these patients.

Hemogram values, although used individually with the normal ranges, are not compared because they were provided from hematology laboratories from several hospitals that used different instruments and that had different reference ranges. Similarly, we did not have consistent information about chemical studies for iron deficiency.

Statistical Analysis

The Student *t* test was used for statistical analysis.

Results

We found that 52 consecutive samples from adults were HbE heterozygotes as judged by the HPLC and CE patterns and the CBCs indicating mild microcytosis with a normal red cell distribution width. The mean HbA_2 of patients with HbE trait was 3.4% (SD, 0.4%), which was significantly higher ($P < .001$) than the 2.6% (SD, 0.4%) for the control group **Figure 1**. Because the HPLC values for HbE included the HbA_2 values, a direct comparison could not be made. However, when the HbA_2 value obtained by CE is added to the HbE values by CE, there is a significantly higher value of the combination by CE (mean, 28.3%) compared with HPLC (mean, 24.5%) ($P < .001$).

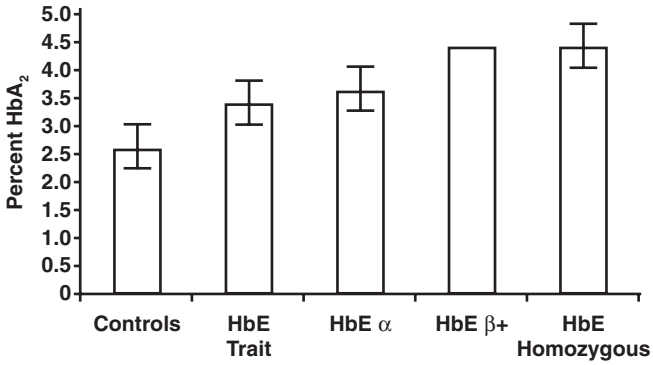

Figure 1 Hemoglobin (Hb) A_2 in HbE cases. The data are presented as mean percentage ± 1 SD. Only 1 case of HbE β+-thalassemia was present.

DOI: 10.1309/AJCPP50JIXXZVLSS

Seven samples were assayed from subjects who were homozygous for HbE. Their mean HbA_2 was 4.4% (SD, 0.4%), which was significantly greater ($P < .001$) than the HbA_2 values for HbE heterozygotes (Figure 1). Four patients were considered doubly heterozygous for HbE and α-thalassemia because their percentage of HbE (mean, 15.8%) was less than 3 SD from patients with HbE trait alone (mean, 25.0%; SD, 2.2).[38] Two patients were considered doubly heterozygous for HbE and β-thalassemia trait because their percentage of HbE (mean, 52.6%) was greater than 3 SD above that of patients with HbE trait only[38] owing to the diminution of HbA production. People with β-thalassemia trait and HbE have increased levels of HbE with decreased HbA levels. The clinical disease in such cases depends on the severity of the β-thalassemia trait.[39]

As shown in Figure 1, the percentage of HbA_2 found in these forms of HbE varies directly with the number of *HbE* genes and the corresponding effect of coexisting α- or β+-thalassemia (Figure 1).

Discussion

CE has the ability to completely separate HbA_2 from HbE,[24,35,37] a distinction possible by only one of the currently available clinical methods of HPLC[25] and not possible by traditional gel electrophoresis.[32] The performance of the CE technique for this purpose has been evaluated previously.[24] While other more laborious techniques have been described for assessment of HbA_2 in the presence of HbE,[40] the Sebia CE and Tosoh HPLC provide this information directly and are suitable for widespread use.[24,25]

The data from our 65 cases provide an estimate of the range of HbA_2 in HbE heterozygotes evaluated by CE. With regard to the proportion of HbE in patients with the HbE trait, Fucharoen and Winichagoon[15] reported a range of 29.4% ± 2.3%; our range for HbE is lower because they included HbA_2 in their measurements, and the present CE technique separates HbA_2 from HbE. We found the range of HbA_2 in HbE heterozygotes to be 3.4% ± 0.4%. These findings confirm the recent findings by Winichagoon et al,[26] who reported the HbA_2 level to be 3.5% ± 0.4% in HbE heterozygous.

The higher percentage resulting from combining the HbE and HbA_2 by the CE technique in our study compared with our HPLC technique likely relates to the underestimation of HbE due to separation of the glycated fraction of HbE by the HPLC technique (the glycated fraction in that technique is included with HbA). However, the CE technique does not separate glycated or other posttranslational products, thereby providing a more complete measurement of HbE as reported previously for HbS and HbC.[24]

The increased percentage of HbA_2 in patients heterozygous for HbE may be explained by the β-thalassemic nature of the *HbE* mutation. The process of substitution of lysine for glutamic acid at position 26 of the β-globin molecule not only produces a hemoglobin molecule with a different electrophoretic migration but also activates a cryptic splice site resulting in decreased production of the variant β chain, in effect a form of β-thalassemia.[41] Indeed, when HbE is coinherited with β^0-thalassemia, severe disease can result, similar to β-thalassemia major.[41]

Because even mild β-thalassemia traits are associated with an increased percentage of HbA_2 owing to increased production of the δ chain and decreased production of the β chain, it is not surprising that a similar effect can be demonstrated in cases of HbE trait. This supposition is supported by the further increase in HbA_2 noted in the 7 patients who were homozygous for HbE. Too few subjects with thalassemia were included to draw specific conclusions, but one would expect that coinheritance of β-thalassemia trait would increase the percentage of HbA_2, as seen in our 2 cases. With doubly heterozygous HbE and α-thalassemia, the relative decrease of the α-globin produced should favor the binding of the normal δ chains over the variant β-globin, resulting in an increase in the percentage of HbA_2, similar to the increase in the percentage of HbA observed in these patients. Larger studies of the latter situations will be needed to confirm these findings.

Because the laboratory is an off-site laboratory, we did not have consistent information about the iron status of the patients with HbE. However, iron deficiency decreases HbA_2 levels, so if it were present to any significant extent in our population, our data would underestimate the increase in HbA_2.[42,43] More difficult is the issue of possible inclusion in the HbA_2 of small amounts of hemoglobin breakdown products. Our previous report demonstrated that HbA_2 is significantly more elevated in patients with heterozygous HbS when measured by HPLC (3.9% ± 1.1%) (where glycated forms are known to coelute with HbS) than when measured by CE (HbA_2, 3.1% ± 0.8%).[24] However, as noted previously, HbA_2 was significantly greater in subjects heterozygous for HbS than in control subjects (HbA_2, 2.6% ± 0.4%) measured by CE.[24] HbS β-globin chains have less avidity for normal α-globin chains than do normal β-globin chains.[44] The modest increase observed in HbA_2 in the HbS trait cases may reflect the slightly increased avidity of the normal δ chains for the α chains compared with the HbS β chains. The somewhat greater increase in HbA_2 we and others have observed in patients with HbE could reflect a similar competition of HbE β chains and/or the aforementioned β-thalassemic effect.

Regardless of the underlying mechanism, the measurement of HbA_2 in the presence of HbE by Sebia CE for the evaluation of hemoglobin variants is advantageous because heterozygotes with a percentage of HbA_2 outside this range

DOI: 10.1309/AJCPP50JIXXZVLSS

(3.4% ± 0.4%) can prompt evaluation for additional abnormalities in hemoglobin production, especially α- or β-thalassemia. Further study is required to delineate the features that permit confident diagnoses of combined HbE-β-thalassemia based on combined RBC indices and the percentage of HbA_2.

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DOI: 10.1309/AJCPP50JIXXZVLSS

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DOI: 10.1309/AJCPP50JIXXZVLSS