Is Hemoglobin Instability Important in the Interaction Between Hemoglobin E and β Thalassemia?

By D.C. Rees, J.B. Clegg, and D.J. Weatherall

Hemoglobin E (HbE; αβ2α2β6glu-lys), globally the commonest hemoglobin variant, is synthesized at a slightly reduced rate and has a homozygous phenotype similar to heterozygous β thalassemia. Yet, when it is inherited together with a β thalassemia allele, the resulting condition, HbE/β thalassemia, is sometimes characterized by a severe, transfusion-dependent thalassemia major. The severity of this interaction has not been explained. We have explored the possibility that it may reflect the instability of HbE consequent upon globin chain imbalance imposed by the β thalassemia allele. Time-course and pulse-chase globin chain synthesis studies at 37°C on peripheral blood and bone marrow suggest that hemoglobin instability is not significant in steady-state HbE/β thalassemia; this is confirmed by density-gradient centrifugation studies that show no decrease in HbE levels relative to HbA as HbE/β thalassemia red blood cells age. Globin binding to membranes was assessed and only α globin chains were found, in contrast to other unstable hemoglobin in which both α and β chains were present. However, in experiments performed on blood from HbE/β thalassemics in the temperature range 39°C to 41°C, there was evidence of instability of HbE, a finding that was also observed in homozygous HbE. These findings suggest that the phenotype of HbE/β thalassemia is primarily the result of the interaction of two β thalassemia alleles; however, hemoglobin instability may be important during febrile episodes, contributing to worsening anemia.

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Materials and Methods

Subjects. Blood was obtained from individuals resident in the UK who were known to have HbE/β thalassemia; none of them had received blood transfusions in the preceding 6 months. Control samples were collected from relatives of the HbEβ thalassemics, β thalassemia intermedia patients (not involving HbE), and patients with known hemoglobin instability. Informed consent was obtained in each case.

Hematologic analysis. Blood was collected into EDTA. Full blood counts were performed using an automated counter. Blood films, reticulocyte stains, and hemoglobin isoelectric focusing were performed using standard techniques.

DNA analysis. DNA was isolated from peripheral blood leucocytes.

Globin-chain biosynthesis. Approximately 20 mL of venous blood was taken into heparin and kept on ice until analyzed, a maximum of 2 hours. The cells were washed three times in cold reticulocyte saline (0.13 mol/L NaCl, 0.005 mol/L KCl, 0.007 mol/L MgCl₂) before ultracentrifugation at 100,000 g to concentrate the reticulocytes. White blood cells were removed from the top 0.6 mL of concentrated red blood cells using α-cellulose and microcrystalline cellulose (Sigmacoll 50; Sigma Chemical Co, Poole, Dorset, UK). This reticulocyte-rich fraction was added to a plasma-based incubation medium and, after 10 minutes of preincubation, 200 µCi of L-[4,5-3H] leucine (Amersham International plc, Little Chalfont, Bucks, UK) was added and the incubation was continued for 2 hours. Incubation temperatures of 37°C, 39°C, and 41°C were used. A bone marrow aspirate sample taken for diagnostic reasons from an HbE/β thalassemic was also studied.
Density gradient centrifugation. The effect of ageing on the hemoglobin composition of circulating red blood cells was analyzed by density gradient centrifugation. Washed erythrocytes from an untransfused HbE/β-thalassemic were centrifuged at 35,000g on a 20% Percoll (Pharmacia, St. Albans, UK)/meglamine gradient for 20 minutes.\textsuperscript{19} The red blood cells were divided into three fractions: a denser older fraction, an intermediate fraction, and a less dense, younger fraction. These fractions were then washed three times in reticulocyte saline and the hemoglobin composition of each was assessed by cation exchange high-performance liquid chromatography (HPLC). Pyruvate kinase (PK) and glucose-6-phosphate dehydrogenase (G6PD) levels were also measured in each fraction to confirm that the age of the cells increased with density.\textsuperscript{20}

RESULTS

Time-course globin-chain synthesis studies at 37°C. Time-course studies at 37°C were performed over 2 hours on 5 individuals with HbE/β-thalassemia; DNA analysis showed the β thalassemia mutations present to be 2 cases of IVS 1-5 (G-C) (severe β\textsuperscript{+}), Fr 8/9 (β\textsuperscript{−}), IVS 1-130 (β\textsuperscript{+}), and IVS II-654 (β\textsuperscript{−}). The steady-state hemoglobin levels ranged from 6.6 to 10.4 g/dL (mean, 8.0 g/dL). Linear synthesis occurred in each case over the period of the incubation, suggesting that HbE is not significantly unstable over 2 hours (Fig 1). The mean ratio of (SA increase of β\textsuperscript{+} in the first hour)/(SA increase in β\textsuperscript{−} the second hour) (SAβ 1 hour/SAβ 2 hours) was 0.94 (range, 0.7 to 1.1; Fig 2). A ratio of 1 results from linear synthesis with no globin instability. This result was confirmed in a sample of bone marrow from an HbE/β-thalassemic [IVS 1-5(G-C)] and using whole, unwashed, blood [IVS 1-5(G-C)], with SAβ 1 hour/SAβ 2 hours ratios of 1.1 and 0.92, respectively; the latter experiment was performed to ensure that washing the cells did not remove any small molecules that might be important in exacerbating HbE instability, eg, free iron and hydrogen peroxide.\textsuperscript{21}

Pulse-chase studies at 37°C. A pulse-chase incubation at 37°C was performed on an HbE/β-thalassemic (IVS 1-5 G-C) over 30 minutes to detect any early hemoglobin instability (Fig

\begin{align*}
\text{Density gradient centrifugation.}\end{align*}
Thalassemia intermedia. The log(SA unwashed, whole blood); EE, homozygous HbE; TI, thalassemia E/beta thal, E/ 

Fig 2. Summary of the results of time-course globin chain synthesis studies. Results are expressed as the ratio of the increase in specific activity of the β or β' chain in the first hour to the increase in the second hour. The log10 of the ratio is plotted. If SA increases linearly, the ratio is 1 (log10 0); as the hemoglobin becomes unstable and the SA increases less in the second hour, the ratio increases exponentially. (•) Incubation at 37°C, (○) incubation at 39°C, (□) incubation at 41°C. (□) sample was also deficient for pyrimidine 5’ nucleotidase. E/β thal, E/β thalassemia (includes bone marrow sample and unwashed, whole blood); EE, homozygous HbE; TI, thalassemia intermedia. The log(SA): 1 hour/SA 2 hours) is 0 in HbE/β thalassemia at 37°C, but increases markedly at higher temperatures. This effect is not seen in β thalassemia intermedia.

Membrane studies at 37°C. Pulse-chase incubations were performed over 12 hours using blood from an HbE/β thalassemic (codon 16, -C). At 30 minutes, a radioactive peak corresponding to newly synthesized α globin was found to be bound to the membrane; no β' chain was observed. At 2 hours, a similar pattern was found with slightly less radioactive incorporation in the α globin peak. By 12 hours, no radioactive globin was found bound to the membrane (Fig 4). The pattern of globin binding contrasts to the findings when 60 minutes of incubation were performed on blood from a known unstable hemoglobin, Hb Bristol (β67 Val-Met—Asp), when both α and β globin chains were found on the membrane (Fig 5). This pattern of membrane-bound globin chains was also observed with Hbs Sun Prairie and Ann Arbor (data not shown).

Globin synthesis studies at 41°C. The 2-hour time-course incubations were repeated at 41°C on the HbE/β thalassemics with the Fr 8/9 and IVS 1-130 mutations. At the higher temperature, there is a marked decrease in the rate of increase of specific activity, typical of hemoglobin instability (Fig 1); this pattern is similar to that seen with unstable hemoglobins at 37°C. Two time-course incubations of HbE/β thalassemia at 39°C also showed a similar plateau. The geometric mean of SAB 1 hour/SAβ 2 hours at higher temperatures was 25 (range, 2.2 to 792; Fig 2), showing a marked increase compared with incubations at 37°C; this wide range reflects the fact that, as the SA increase in the second hour decreases towards zero, the ratio increases exponentially. This contrasted to a control experiment in which blood from an untransfused patient with thalassemia intermedia (Cd39/IVS 1-6) showed linear increases in specific activity with time at both 37°C and 41°C (SAβ 1 hour/SAβ 2 hours ratios at 1.6 and 1.9 at 37°C and 41°C). Both β and γ increased relative to α. This temperature effect has been noted before, but its explanation and significance are unclear.

The pattern of hemoglobin binding to the membrane in HbE/β thalassemia at 41°C was similar to that at 37°C, with α globin but no β' chains bound. Studies on HbE homozygotes. Parallel experiments were performed on the red blood cells of a HbE homozygote. Time-course studies at 37°C showed linear synthesis as expected, but increasing the temperature to 41°C showed the development of a plateau, similar to that in HbE/β thalassemia. The SAβ 1 hour/SAβ 2 hours ratio increased from 0.7 at 37°C to 18 at 41°C. In contrast to HbE/β thalassemia, membrane analysis showed little globin binding at 37°C and 41°C, although at the higher temperature there was a small peak in the β' position.

Density gradient centrifugation. Density-gradient-separated fractions of blood from an HbE/β thalassemic [IVS 1-5(G-C)] were analyzed for hemoglobin composition, with the centrifugation and chromatography being performed in duplicate. The results are summarized in Fig 6. There is a relative increase in HbF, mirrored by a decrease in Hbs E and A in the older, denser fraction. The HbF/HbA ratio increased from 5.3 in the younger fraction to 9.2 in the older. On the other hand, there was no significant change in the HbE/HbA ratio across the
gradient, indicating that there was no loss of the HbE relative to HbA during the lifespan of red blood cells. The pyruvate kinase activity decreased from 9.6 IU/10^10 red blood cells in the top fraction to 4.15 in the bottom; G6PD levels fell similarly from 3.71 IU to 2.44.

**DISCUSSION**

This is the first study to address directly the question of HbE instability in its most important clinical context, HbE/β thalassemia. There is little previously published evidence either for or against the clinical importance of the instability of HbE in vivo. There is a single case report of dapsone causing a Heinz body hemolytic anemia in a Cambodian woman with HbE trait,23 although this has not been confirmed by subsequent observations, despite the widespread use of the drug in areas where HbE is prevalent. A second study indirectly implicated HbE instability as important in protection against malaria, by noting that HbE only offered protection if fava beans had been consumed in the preceding month.24 We have shown previously that pyrimidine 5’ nucleotidase deficiency interacts with HbE to produce hemoglobin instability and hemolysis.11 However, there are a large number of reports of HbE occurring with other hemoglobin and red blood cell defects, without any suggestion of interaction: these include HbE with HbS,25 HbC,26 Hb Lepore,27 Hb Hope,28 G6PD deficiency,29-31 and hereditary elliptocytosis.32,33 The interaction between HbE and HbH disease is particularly interesting: the resulting condition, HbAE-Bart’s disease, is similar in severity to HbH disease,28 suggesting that
the HbE does not ameliorate the condition by reducing globin chain imbalance or exacerbate it by maximizing the hemolytic component.

The pattern of globin binding to the red blood cell membrane at 37°C is similar to that found in thalassemia major, with only α globin present, and has been confirmed by electron microscope immunocytochemical studies of inclusions in HbE/β-thalassemia. Time-course studies show that newly bound α globin is proteolysed within 2 and 12 hours of precipitation. This suggests that the amount of globin bound to the membrane in β thalassemia is determined by a dynamic equilibrium between the rate of precipitation and the rate of hemolysis and indicates that the majority of globin-induced damage to the membrane may occur late in development of the red blood cell, because the proteolytic capacity of the reticulocyte is very limited compared with that of the earlier nucleated red blood cell precursors.

We have found no evidence that instability of HbE is important in the steady state in HbE/β-thalassemia. Time-course globin chain synthesis experiments showed linear synthesis at 37°C in both marrow and peripheral blood. Pulse-chase studies showed that only α globin, not βE globin, binds to the red blood cell membrane, in contrast to other unstable hemoglobinics such as Hb Bristol.

Density centrifugation of red blood cells from a HbE/β-thalassemic supported these findings (Fig 6). There is good evidence that in most conditions red blood cells become more dense as they age; sickle cell syndromes appear to be the exception to this. In our experiment, the decreasing levels of PK and G6PD activity associated with increasing density strongly suggest that the denser fractions are predominantly made up of older cells. The relative increase in HbF with cell age reflects the well-defined survival advantage of cells containing more HbF, but there was no significant loss of HbE relative to HbA in the older cell population. Taken together, these data suggest that HbE is not significantly unstable in vivo in the steady state.

However, the globin chain synthesis studies at higher temperatures suggest that newly synthesized hemoglobin is unstable in HbE/β-thalassemia, in contrast to the effect of increasing temperature on control cells from a patient with β thalassemia intermedia. The instability involved α, βE, and γ chains, suggesting that hemoglobin tetramers containing βE chains are precipitated, rather than free globin chains, a situation that has been observed previously with unstable hemoglobins. Instability occurred at both 39°C and 41°C, both significantly above body temperature; however, in tropical regions where malaria is endemic, febrile illnesses are common and the resulting exacerbation of dyserythropoiesis and hemolysis might be a significant cause of variability in hemoglobin levels in cross-sectional studies. It is known that fevers cause increased hemolysis in patients with unstable hemoglobins and hemoglobin H disease. There is an anecdotal report that fevers cause a more marked decrease in hemoglobin in HbE/β thalassemia and HbE disease, but this potentially important observation has not been studied subsequently.

Are there other possible interpretations of the result of the globin chain synthesis studies at higher temperatures? One possible explanation for the plateau in the rate of increase of specific activity is that the rate of mRNA translation starts to decrease over 2 hours at 41°C; the maintenance of linear synthesis in thalassemia intermedia at 41°C goes against this. Although the hemoglobin appears to be unstable at higher temperatures in HbE/β-thalassemia, the pattern of globin binding to the membrane is unchanged compared with that at 37°C, with only α globin found. This suggests that precipitated βE chains are either proteolysed rapidly from the membrane or that they are unable to bind to the membrane at all. This absence of β globin binding to the membrane is similar to that in the interaction between HbE and pyrimidine 5′ nucleotidase deficiency. It is also interesting that the HbE homozygote showed a similar temperature effect to HbE/β thalassemics, with a plateau occurring in specific activity after 1 hour at 41°C and an SAB 1 hour/SAB 2 hours ratio of 18; it differed, however, in that no definite detectable peak of radioactivity was found attached to the membrane. The clinical correlate of this finding in homozygous HbE is unclear, with little information on the effects of fever on homozygotes.

This study suggests that the nature of the interaction between HbE and β-thalassemia is primarily the interaction of two β thalassemia alleles and that the resulting degree of anemia is related to the amount of globin chain imbalance. Similarly, the marked variability in severity between different individuals with HbE/β-thalassemia principally results from differences in globin chain imbalance, including variability in γ chain synthesis and coexistent α-thalassemia. However, we have also found evidence that HbE can be significantly unstable when subjected to mild oxidative stress, such as increases in body temperature, and this may also contribute to the variability and severity. The significance of these temperature effects needs to be addressed in clinical studies.
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