The Unusual Pathobiology of Hemoglobin Constant Spring Red Blood Cells

By S.L. Schrier, A. Bunyaratvej, A. Khuhapinant, S. Fucharoen, M. Aljurf, L.M. Snyder, C.R. Keifer, L. Ma, and N. Mohandas

Hemoglobin Constant Spring (HbCS) is the most common nondeletional α-thalassemic mutation and is an important cause of HbH-like disease in Southeast Asia. HbCS variants have an almost normal mean cell volume (MCV) and the anemia is more severe when compared with other α-thalassemic variants. We explored the pathobiology of HbCS red blood cells (RBCs) because the underlying cause(s) of this MCV “normalizing” effect of HbCS and the more severe anemia are not fully explained. HbCS containing RBCs are distinctly overhydrated relative to deletional α-thalassemia variants, and the derangement of volume regulation and cell hydration occurs early in erythroid maturation and is fully expressed at the reticulocyte stage. Furthermore, the membrane rigidity and membrane mechanical stability of HbCS containing RBCs is increased when compared with HbH and α-thalassemia-1 trait RBCs. In seeking the cause(s) underlying these cellular alterations we analyzed membranes from HbCS and deletional α-thalassemic variants and found that in addition to oxidized β-globin chains, oxidized α- or γ-globin chains are also associated with the membranes and their skeletons in HbCS containing RBCs. We propose that the membrane pathology of HbCS variants is caused by combination of the deleterious effects induced by membrane-bound oxidized α- or γ-globin chains. The membrane alterations induced by α- or γ-chains are more akin to those induced by β-globin chains than those induced by the α- or γ-globin chains that accumulate in the β-thalassemias. Thus, each globin chain, α, α, γ, or β, appears to produce its own form of membrane perturbation.

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AID Blood 0004 / 5H2F$SS$61 01-31-97 09:28:48 bldas WBS: Blood
shipped on ice to California. Each shipment was accompanied by blood drawn from at least one control subject in exactly the same manner and under exactly the same conditions. Analysis of RBC indices were done at the Ramathibodi Hospital, measurement of membrane deformability and stability were performed at the Lawrence Berkeley National Laboratory, and all other analyses were performed at Stanford University.

**Determination of RBC volume, Hb content, and Hb concentration.**

The Bayer H*3 automated hematology analyzer (Diagnostics,Tar- and biffreticulocytes freshly delivered from the bone marrow already show forming these studies we analyzed 11 normal shipment controls, 3 normal limits can also be determined.17 Furthermore, reticulocytes Thiol-disul®de exchange chromatography was performed to iden-

concentration, and Hb content of individual RBCs and displays these were revealed by a peroxidase-conjugated rabbit anti-mouse Ig ob-

life span of RBCs in the peripheral circulation. perinormal RBCs.

**Membrane mechanical stability and deformability measurements.**

Resealed membranes were prepared for mechanical stability and deformability measurements as previously described.2 The erythrocytes were washed three times in 5 mmol/L Tris, 140 mmol/L NaCl (pH 7.4), and then lysed in 40 vol of 7 mmol/L NaCl and 5 mmol/L Tris (pH 7.4). The membranes were then pelleted by centrifugation, resuspended in 10 vol of 5 mmol/L Tris and 140 mmol/L NaCl (pH 7.4), and incubated for 30 minutes at 37°C for rescaling.

For mechanical stability measurements, the ressealed membranes were pelleted by centrifugation and 100 μL of a 40% membrane suspension was mixed with 3 mL dextran (40,000 molecular weight, 35 g/100 mL in 10 mmol/L phosphate buffer, pH 7.4, viscosity 95 cp) and subjected continuously to 750 dynes/cm² in the ektacytometer. Under this stress, the membranes progressively fragment, generating undeformable spheres. This process is detected as a time-

dependent decrease in the Deformability Index (DI). The time re-

quired for the DI to decrease to 60% of its maximum value is termed T60 and is taken as a measure of mechanical stability.2 For deformability measurements, ressealed membranes, prepared as described above, were suspended in 3 mL of Stractan (Arabinogalactan, St Regis Paper Co, Tacoma, WA) (290 mOsm, 22 cp, pH 7.4) and exposed to gradually increasing shear stress (0 to 125 dynes/cm²) in the ektacytometer. For ressealed membranes, the shear stress required to obtain a defined value of DI is determined by the property of membrane deformability without contributions from either internal viscosity or cell geometry. Analysis of the DI curve generated by the ektacytometer provides a measure of membrane deformability.2 These studies were performed on 4 patients with α-thalassemia-1 trait, 3 with HbCS trait, 2 with homozygous HbCS/CS, 4 with HbH, and 3 with HbH/CS.

**Analysis of ghosts and membrane skeletons.**

RBCs were washed extensively and then pretreated with proteolysis inhibitors diisopropyl fluoro-phosphate (DFP, Sigma, St Louis, MO) (2 mmol/L), pep-

tatin A (10 mg/mL), and leupeptin (10 mg/mL) for 30 minutes at 37°C. Ghosts were then prepared by lysing 1 vol of packed erythrocytes with 40 vol of lysing buffer consisting of 5 mmol/L phosphate buffer (pH 8.0) containing 0.5 mmol/L DFP. The membranes were then pelleted by centrifugation and washed three times with the lysing buffer.2,19 Membrane skeletons were prepared from the washed ghosts by Triton extraction exactly as previously described.2,19

**RESULTS**

**RBC parameters.**

The Hb values, RBC indices, and proportions of hypochromic RBCs in the different variants of α-thalassemia and HbCS are summarized in Fig 1. The degree of anemia as reflected by Hb levels ranged from being very mild in individuals with HbCS trait to being quite severe in individuals with HbH/CS. In general, the extent of decrease in Hb values was as follows: HbH/CS > HbH > HbCS/CS > α-thalassemia-1 trait > HbCS trait. As expected, the mean cell Hb content of RBCs in these variants was decreased. The smallest decrease in Hb content was noted in HbCS trait while the largest decrease was found in HbH and HbH/CS. RBCs in α-thalassemia-1 trait and HbCS/CS exhibited intermediate decrease in Hb content. The largest decrease in cell volume was seen in α-thalassemia-1 trait and in HbH and the extent of decrease in cell volume was roughly proportional to the decrease in Hb content. Thus, the mean cell Hb concentration of α-thalassemia-1 trait RBC was only slightly decreased compared with that of normal RBCs. In contrast, RBCs in HbH and HbH/CS had larger cell volumes in relation to their Hb content, res-

ting in marked reduction in the mean cell Hb concentration. RBCs in Hb CS/CS also showed considerable decrease in mean cell Hb concentration. The discordance between cell volume and cell Hb content in these variant RBCs is best illustrated by quantifying the numbers of hypochromic RBCs (Fig 1, bottom panel). Although less than 1% of normal RBCs are hypochromic (Hb concentration values <28 g/dL), approximately 70% of RBCs in HbH/CS are hypo-

chromic. The percentage of hypochromic RBCs proceeded in the following order: HbH/CS > HbH > HbCS/CS > α-thalassemia-1 trait > HbCS trait. The difference between α-thalassemia-1 trait and HbCS trait was not significant. These results imply that RBCs in these different variants are unable to regulate their volume in accordance with their Hb content, thereby leading to increased hydration. These results further suggest that α∗+ may have distinct effects on RBC volume regulation.
are shown in Table 1. A distinguishing feature of these histograms is that reticulocytes in both HbH and HbH/CS exhibit increased volume and decreased cell Hb concentration compared with their mature RBC counterparts, a feature that is also characteristic of reticulocytes in normal blood. Thus, the disproportionate increase in cell volume relative to its Hb content is a feature that is expressed even at the reticulocyte stage in these variant RBCs. From these data we can conclude that the deranged volume regulation of RBCs responsible for their increased hydration occurs early in their evolution. The finding that these reticulocytes enter the circulation from the bone marrow already overhydrated and once in circulation are not able to regulate their volume in relation to their Hb content implies that the transport mechanisms responsible for volume regulation of these RBCs has been irreversibly damaged.

The Hb content histograms in HbH and HbH/CS also exhibited an interesting feature. In normal blood, the Hb content of reticulocytes and RBCs is very similar and the distributions are symmetric. In contrast, while reticulocyte Hb content histograms in both HbH and HbH/CS are symmetric, the RBC histograms are markedly asymmetric with a characteristic tail extending to low values of Hb content (Fig 2). This finding implies that during their circulatory life span, but not during reticulocyte maturation, some RBCs underwent fragmentation resulting in generation of mature cells with decreased Hb content. This fragmentation may be relatively greater in α-thalassemia-1 trait and in HbH because these variants showed the greatest reduction in MCV from the reticulocyte stage to mature RBC (Table 1, MCV ratios, respectively, of 1.35 \( P < .05 \) and 1.41 \( P < .0001 \) when compared with the normal).

Membrane material properties. Mechanical stability and dynamic rigidity of resealed membranes prepared from these variant α-thalassemic RBCs was measured by ektacytometry and the data are shown in Fig 3. Membrane dynamic rigidity was increased for the variant RBCs with membranes of HbCS/CS RBCs exhibiting the highest increase in rigidity (Fig 3A). The greatest increase in mechanical stability was noted for membranes of HbCS/CS RBCs, whereas membranes of HbH/CS and HbH RBCs exhibited intermediate increases in membrane rigidity, whereas membranes of α-thalassemia-1 trait RBCs exhibited no increase in rigidity (Fig 3). In addition to increased membrane rigidity, these variant RBCs also exhibited increased membrane mechanical stability (Fig 3B). The greatest increase in mechanical stability was noted for membranes of HbCS/CS RBCs, whereas membranes of HbH/CS and HbH RBCs exhibited intermediate increases in membrane mechanical stability. The membrane mechanical stability of CS trait RBCs and α-thalassemia-1 trait RBCs was similar of that of normal RBCs (data not shown). These findings suggest that α\(^{-}\)-chain interaction with the membrane can induce changes in material properties that mimic the membrane changes induced by excess β-globin chains interacting with the membrane in HbH RBCs.

Analysis of membrane and skeletal protein components. To determine the nature of the globin chains interacting with membranes in these variant RBCs, we performed SDS-PAGE analysis of ghosts from patients with HbH disease,
Fig 2. Parameters for the RBCs and reticulocytes from a normal patient, a patient with classical HbH, and a patient with classical HbH/CS. The distribution curves for the entire RBC population are shown in gray, whereas the reticulocyte population is displayed in red.

Table 1. Comparison of Values in Reticulocytes and RBCs

<table>
<thead>
<tr>
<th></th>
<th>RBCs (mean ± SD)</th>
<th>Reticulocyte (mean ± SD)</th>
<th>Ratio: Reticulocytes/RBCs (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal (10)</td>
<td>89.27 ± 3.89</td>
<td>113.98 ± 4.97</td>
<td>1.28 ± 0.04</td>
</tr>
<tr>
<td>α-T (7)</td>
<td>75.49 ± 4.53</td>
<td>102.00 ± 4.96</td>
<td>1.35 ± 0.08</td>
</tr>
<tr>
<td>CS-T (8)</td>
<td>85.38 ± 4.12</td>
<td>106.38 ± 4.81</td>
<td>1.25 ± 0.03</td>
</tr>
<tr>
<td>CS/CS (6)</td>
<td>82.05 ± 3.72</td>
<td>98.68 ± 5.45</td>
<td>1.20 ± 0.06</td>
</tr>
<tr>
<td>HbH (30)</td>
<td>74.79 ± 7.81</td>
<td>104.89 ± 8.00</td>
<td>1.41 ± 0.10</td>
</tr>
<tr>
<td>HbH/CS (19)</td>
<td>85.74 ± 5.65</td>
<td>107.48 ± 5.81</td>
<td>1.26 ± 0.06</td>
</tr>
<tr>
<td>MCHC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>31.27 ± 0.82</td>
<td>25.65 ± 1.13</td>
<td>0.82 ± 0.03</td>
</tr>
<tr>
<td>α-T</td>
<td>28.03 ± 0.63</td>
<td>22.63 ± 1.35</td>
<td>0.81 ± 0.04</td>
</tr>
<tr>
<td>CS-T</td>
<td>29.81 ± 0.64</td>
<td>25.09 ± 0.64</td>
<td>0.84 ± 0.01</td>
</tr>
<tr>
<td>CS/CS</td>
<td>29.03 ± 1.21</td>
<td>25.53 ± 2.01</td>
<td>0.88 ± 0.04</td>
</tr>
<tr>
<td>HbH</td>
<td>23.94 ± 1.29</td>
<td>19.15 ± 0.86</td>
<td>0.80 ± 0.04</td>
</tr>
<tr>
<td>HbH/CS</td>
<td>22.46 ± 1.22</td>
<td>20.02 ± 1.12</td>
<td>0.89 ± 0.03</td>
</tr>
<tr>
<td>MCH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>27.22 ± 1.66</td>
<td>28.20 ± 1.53</td>
<td>1.04 ± 0.02</td>
</tr>
<tr>
<td>α-T</td>
<td>20.51 ± 1.59</td>
<td>22.27 ± 1.77</td>
<td>1.09 ± 0.05</td>
</tr>
<tr>
<td>CS-T</td>
<td>24.74 ± 1.18</td>
<td>25.96 ± 1.12</td>
<td>1.05 ± 0.01</td>
</tr>
<tr>
<td>CS/CS</td>
<td>23.03 ± 1.60</td>
<td>24.32 ± 1.05</td>
<td>1.06 ± 0.03</td>
</tr>
<tr>
<td>HbH</td>
<td>16.85 ± 1.28</td>
<td>19.31 ± 1.00</td>
<td>1.15 ± 0.05</td>
</tr>
<tr>
<td>HbH/CS</td>
<td>18.27 ± 1.12</td>
<td>20.58 ± 1.06</td>
<td>1.13 ± 0.04</td>
</tr>
</tbody>
</table>

The number in parentheses in the top panel indicates the number of patient samples analyzed.
HbH/CS, and Hb CS/CS and the data are shown in Fig 4 (left panel). The HbCS trait was omitted from this analysis because the band was too faint to be seen regularly. In all of the ghost membranes from patients with HbCS, a polypeptide species migrating with a molecular weight of 19 kD was detected. As $\alpha^a$ with its additional 31 amino acids has a molecular weight of approximately 19 kD, these data suggest that this polypeptide is likely to be membrane-associated $\alpha^a$. To unequivocally document that this polypeptide is indeed $\alpha^a$, Western blotting of these proteins was performed using MoAbs to human $\alpha$- and $\beta$-globin chains, and the data are shown in Fig 4. The 19-kD polypeptide species reacted with the $\alpha$-globin MoAb but not with $\beta$-globin antibody, confirming the identity of the membrane-associated polypeptide as $\alpha^a$. As we have previously shown, only $\beta$-globin chains were found in association with HbH membranes (Fig 4). Interestingly, the amount of membrane-bound globin was substantially increased in two splenectomized patients, one with the HbH genotype and the other with HbH/CS genotype, compared to membranes from their non-splenectomized counterparts. Using laser densitometry we were able to measure the ratio of spectrin to $\alpha^a$ in two patients with HbH/CS and one patient with Hb CS/CS. Using relatively standard assumptions regarding membrane protein content, we were able to calculate that there were 0.16 and 0.25 mg of membrane-associated $\alpha^a$ per $10^{10}$ RBCs in HbH/CS and 0.4 mg of $\alpha^a$ per $10^{10}$ RBCs in the patient with HbCS/CS. Assuming that the cytosolic HbCS content is about 2% in HbH/CS and about 4% in Hb CS/CS, then about 7% of $\alpha^a$ is membrane bound in each case.

We had previously shown that much of the membrane-bound $\alpha$-globin in severe $\beta$-thalassemia and $\beta$-globin in $\alpha$-thalassemia is associated with membrane skeletons. To determine if membrane-associated $\alpha^a$ chains are also associated with membrane skeletons, we performed thiol-disulphide exchange chromatography, and the data are shown in Fig 6. In this assay, the unbound fraction represents proteins devoid of free thiols and, hence, oxidized proteins. The unbound protein fraction of membranes prepared from $\alpha^a$ variant RBCs were analyzed by SDS-PAGE. The 19-kD polypeptide corresponding to the molecular weight of $\alpha^a$ was indeed found to be associated with membrane skeletons in all HbCS variant RBCs (Fig 5) except HbCS-T, where the amounts were too small to be seen regularly. We then determined by laser densitometry the ratio of spectrin to $\alpha^a$ in ghosts and skeletons in a patient with Hb CS/CS and found that 55% of membrane-bound $\alpha^a$ remained with the skeleton fraction.

To determine if there had been oxidation of membrane-associated globin chains, we performed thiol-disulphide exchange chromatography, and the data are shown in Fig 6. In this assay, the unbound fraction represents proteins devoid of free thiols and, hence, oxidized proteins. The unbound protein fraction of membranes prepared from HbCS/CS cells contained $\alpha^a$-globin chains, indicating that the membrane-associated $\alpha^a$-globin chains were indeed oxidized. Membranes of HbH RBCs contained only oxidized $\beta$-globin chains. In Fig 6, $\beta$-thalassemia intermedia membranes contained only oxidized $\alpha$-globin chains. All HbCS-containing RBCs had varying amounts of partially oxidized membrane-associated $\alpha^a$ (not shown). Using laser densitometry we determined the ratio of $\alpha^a$ to glycophorin A in two patients with Hb CS/CS from which we calculated that 15% to 30% of the membrane-associated $\alpha^a$ had become oxidized. This probably means that the single cysteine at position 104 had become oxidized.

**DISCUSSION**

The HbCS gene is very common in Southeast Asia and its frequency approaches 5% to 8% in Thailand. The CS mutation only affects the $\alpha_2$ gene, which accounts for about 2/3 of normal $\alpha$-globin chain production. Furthermore, the...
mRNA of \( \alpha^{+} \) is very unstable compared with normal \( \alpha \) mRNA and as such accounts for less than 1% of protein output of a normal \( \alpha \) gene. Interestingly, in terms of pathophysiology, the synthesis of even small amounts of elongated \( \alpha^{+} \) results in more severe anemia than is seen in analogous deletional \( \alpha \)-thalassemia-2. Therefore, it has been suggested that \( \alpha^{+} \)-chains may have deleterious effects on cellular and membrane properties of HbCS-containing RBCs and these changes in turn could account for increased hemolysis. To date the only well-documented pathobiologic feature of some of the HbCS variant RBCs is their increased cell volume relative to their Hb content. The unique properties of HbCS lead to studies indicating that the inclusion bodies seen in HbH are distributed somewhat differently than the inclusion bodies seen in HbH/CS. There is defective spectrin self association in HbH/CS but the same pattern is seen in HbH. HbCS/CS skeletons dissociated more readily with release of spectrin, actin, and band 4.1 than skeletons of HbH/CS which in turn dissociated more readily than HbH. Other than these observations there is currently little information available on various membrane alterations of HbCS-containing RBCs and the mechanistic basis for these changes.

The present study has enabled us to document a number of cellular and membrane changes in HbCS variant RBCs and also obtain some new insights into the mechanistic basis for these changes. A distinguishing feature of all HbCS variant RBCs studied is their increased volume relative to their Hb content (Fig 1), resulting in a large increase in the percentage of hypochromic (Hb concentration <28 g/dL) RBCs (Fig 1). Increased numbers of hypochromic RBCs were seen even in heterozygous HbCS RBCs, implying a role of \( \alpha^{+} \) in cell volume regulation. The increased numbers of hypochromic RBCs in HbH/CS compared with HbH further validates this thesis. Importantly, our finding that reticulocytes in both HbH and HbH/CS are more hydrated than normal reticulocytes (Fig 2, Table 1) implies that hydration of these cells occurs early in their development. We have previously documented increased hydration of HbH RBCs and proposed that damage to membrane transport pathways induced by membrane-associate oxidized \( \beta \)-chains may be responsible for the deranged volume regulation of these RBCs. Our present finding that increased hydration is also a feature of reticulocytes in HbH allows us to consider that damage to the K-Cl cotransport pathway might be the basis for increased hydration of these RBCs without excluding other cellular hydration systems. However, the K-Cl cotransport pathway plays a major role in volume regulation of early erythroid cells as well as reticulocytes; therefore, damage to this transport mechanism could prevent the volume loss that accompanies maturation of normal erythroblasts and reticulocytes to RBCs. Furthermore, the finding that reticulocytes and RBCs in HbH/CS are even more hydrated than comparable HbH RBC suggests that \( \alpha^{+} \) interacting with the membrane may accentuate the damage induced by \( \beta \)-globin chains to the K-Cl cotransport, or other transport systems controlling cellular hydration. These effects of \( \alpha^{+} \) on cell volume regulation resemble those exerted by \( \beta \)-globin rather than those induced by \( \alpha \)-globin. Quantitation of membrane rigidity and mechanical stability showed that HbCS variant RBCs demonstrated considerable alterations in these membrane material properties.
creased membrane rigidity and increased membrane mechanical stability were features of all the HbCS variant cells studied. These changes are identical in direction to those induced by oxidized β-globin chains interacting with the membrane and enhanced them. Thus, as with volume regulation, the membrane alterations induced by αcs were akin to those induced by β-globin chains rather than those induced by α-globin chains in severe β thalassemia.

The exploration of the biochemical basis for these impressive cellular and membrane alterations in HbCS variant RBCs showed that partially oxidized αcs was associated with the membranes of all HbCS variant RBCs (Figs 4 and 6). Membrane skeletal preparations of HbCS variant RBC revealed that αcs was associated with membrane skeletal structures (Fig 5). Thus, as in the case of HbH and β-thalassemia intermedia where oxidized β-globin and oxidized α-globin, respectively, are associated with the membrane and its skeletons, partially oxidized αcs is associated with the skeletal structure in all the HbCS variant RBCs. We do not know why αcs behaves in this manner. The 31 additional amino acids do not contain an extra cysteine which could be oxidized. But this stretch of amino acids contains 14 relatively hydrophobic amino acids and thus might lead to insertion of αcs into membrane transport sites. By comparing the cellular and membrane alterations documented in HbH, HbH/CS, HbCS/CS, and β-thalassemia intermedia, we come to the conclusion that oxidized αcs induced changes that mimic those induced by oxidized β-globin.

The mechanism(s) by which these highly specific globin-chain-induced abnormalities are produced remains incompletely understood. Each of these globin chains contains

Fig 6. Thiol-disulfide exchange chromatography was performed on a normal control, a β-thalassemia intermedia patient who had been splenectomized, an HbH patient who had been splenectomized, and a patient with homozygous HbCS/CS. The unbound fraction was analyzed by SDS-PAGE and the αcs was identified at a position consistent with a molecular weight of 19 kD. Note the presence of globin bands at the bottom of the gel. These have previously been shown to be αcs in β-thalassemia intermedia and β in HbH.
HEME and its associated iron. The role of this membrane-
associated iron in producing localized oxidant damage in
sickle disease and in thalassemia has been extensively ex-
plained by Shalev et al.25 A likely hypothesis is that each of
these partially oxidized globin chains (αα, αβ, and ββ) pro-
duce highly specific sorts of oxidant damage relating to their
underlying physical chemistry and special sites of localiza-
tion. We believe that we have begun to unravel the unique
pathophysiology of the HBs variants that accounts for the
increased severity of the anemia and the related alterations in
cellular hydration and in membrane mechanical proper-
ties. In these HBs variants of α-thalassemia, in addition to
the membrane accumulation of the excess β-globin chains
characteristic of all α-thalassemias, there is membrane-asso-
ciated αα as well. Surprisingly, this additional impact pro-
duced by αα leads to membrane damage more akin to that
produced by ββ than the αα accumulation seen in β-thalas-
semia. The mechanistic basis for the effects of oxidized αα
on cell volume regulation and membrane material properties
awaits further detailed studies of the specific protein targets
perhaps undergoing oxidant damage, including the K-Cl
transporter, spectrin, and band 3.

NOTE ADDED IN PROOF

While the manuscript was being prepared Prof Prapon
Wilairat informed us that he had also noted the presence of
αCS in the membrane of RBC containing HbCS.26

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αCS-globin on membrane of red cells containing hemoglobin constant
The Unusual Pathobiology of Hemoglobin Constant Spring Red Blood Cells

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