Hemichromes in Single Inclusion Bodies in Red Cells of Beta Thalassemia

By Eliezer A. Rachmilewitz and Bo Thorell

Inclusion bodies in the red cells of patients with thalassemia syndromes may result from precipitation of those hemoglobin subunits that are produced in relative excess. In hemoglobin H disease, a form of a-thalassemia, the inclusions are precipitated \( \beta \)-subunits, while in the \( \beta \)-thalassemias they are \( \alpha \)-subunits. It has been shown that the inclusions in hemoglobin H disease have the spectral characteristics of hemichrome. In the present study, microspectrophotometric examination of single inclusion bodies in ghosts prepared from the red cells of two patients with \( \beta \)-thalassemia major revealed the absorption spectrum of hemichrome. These results suggest that hemichrome formation is an important pathogenetic event in the production of red cell inclusions and consequent hemolysis in the thalassemias.

Previous studies have shown that oxidation of isolated \( \alpha \)- or \( \beta \)-subunits of hemoglobin A results in a transient appearance of ferrihemo-

globin followed by the formation of an unstable hemichrome. Hemichromes are heme proteins in which both the fifth and sixth coordination positions of the heme iron are liganded to nitrogenous bases. In the case of the hemoglobin subunits, the hemichrome is thought to result from bonding of the histidine at helical position E7 to the sixth coordination position, while the normal bond between histidine F8 and the fifth position remains intact.

In hemoglobin H disease, a form of a-thalassemia, free \( \beta \)-subunits are present in the red cells as a consequence of the impaired synthesis of \( \alpha \)-globin polypeptide chains. The inclusion bodies in the red cells of patients with this disorder were shown to be \( \beta \)-subunits with the optical absorption and electron spin resonance spectral characteristics of hemichromes. These findings suggested that the precipitation of Hb H (\( \beta_4 \)) in the red cells occurred as a result of hemichrome formation.

The present report concerns a microspectrophotometric examination of single inclusion bodies that were induced by incubation of red cells of patients with \( \beta \)-thalassemia. These inclusions also had the spectral characteristics of hemichromes. This finding suggests that hemichrome formation is an important pathogenetic event in both \( \alpha \)- and \( \beta \)-thalassemia.
MATERIALS AND METHODS

Fresh red cells were obtained from the antecubital vein and kept at 4°C until use. One sample was lysed with water, and the ghosts were examined for inclusion bodies by phase-contrast microscopy. In addition, hemoglobin-free ghosts were prepared for microspectrophotometry in 0.001 M EDTA, 0.05 M Tris-HCl buffer pH 7.0 according to the method of Marchesi and Palade. To obtain sufficient amounts of inclusion bodies the patients' red cells had to be incubated for 12 hr at 37°C prior to the preparation of red cell ghosts. The ghost preparations together with samples of fresh blood cells from the same patient were flown from Jerusalem to Stockholm in a thermos flask precooled to 4°C. Control samples of normal red cells were similarly treated for microspectrophotometric studies.

Within 24 hr after collection of the blood and preparation of the ghosts, absorption spectra of red cells and of red cell ghosts with and without inclusion bodies were recorded with a microspectrophotometer modified from the instrument described by Chance et al. In the present work, it was used with a Zeiss Ultrafluar condenser, stopped down to NA 0.6, and an ultrafluar objective 100 X NA 0.85. Most spectra were recorded with a full scale deflection corresponding to an absorbancy of 0.1. When recording the spectra from single inclusion bodies in the red cell ghosts, the scale was further expanded so that full deflection of the record corresponded to an absorbency of 0.01. The spectra were recorded after a smear was prepared, air dried immediately, and then covered with paraffin oil before mounting the cover slip. The inclusion bodies selected by phase-contrast microscopy measured over 1 μ in diameter and were, therefore, large enough to cover the photocell aperture of the microspectrophotometer. Complete absorption spectra were run between 500 μ and 600 μ and also between 500 μ and 400 μ covering the Soret region.

Since it was necessary to use a relatively large scale expansion in order to record the discrete α and β peaks of hemoglobin in these rather "hypochromatic" red cells, the appropriate baseline could not be recorded at the same time but had to be estimated in separate measurements. With the present microoptical system and type of cell preparation, the baseline or "zero" differences between 500 and 600 μ never exceeded 0.01 in absorbancy (Fig. 1).

The present studies were performed on blood samples from two sisters, aged 14 and 17 yr, with β-thalassemia major. The detailed family pedigree of these patients is described elsewhere. Both patients had undergone splenectomy 8 yr previously. Although their

Fig. 1. Absorption spectrum from normal red cell with two absorption peaks of oxyhemoglobin at 576 and 542 μ. Slightly displaced duplicate record indicates reproducibility of the measurements. Dotted line represents slope of baseline.
hemoglobin level was between 6–7 g/100 ml, they did not require blood transfusions and were fairly well adjusted to their low hemoglobin levels. In both patients the reticulocyte count was in the region of 3%. The fetal hemoglobin levels determined by the method of Singer et al.7 were 40% in the older and 20% in the younger sister. Both had elevated levels of hemoglobin A₂ ranging between 5 and 7%, as determined by cellulose acetate electrophoresis.8

RESULTS

When the whole blood of the patients was incubated at 37°C for 12 hr followed by lysis of the washed red cells, the color of the ghosts remained brownish red, in spite of repeated washing with Tris-HCl buffer pH 7.0. Phase-contrast microscopy revealed inclusion bodies of various sizes in the lysed red cells from both patients (Fig. 2). Ghosts of similarly treated normal red cells were colorless, and no inclusion bodies were seen.

When the thalassemic red cells were examined at a wave length close to the Soret band region, the degree of hemoglobinization in various parts of the single red cell appeared very nonhomogenous. Inclusion bodies in intact red cells stood out as heavily absorbing, whereas, other parts of the cell were apparently almost hemoglobin free.

The red cell ghosts were nonabsorbing from 700 to 400 mμ, except for the inclusion bodies that appeared to have considerably less absorption than the inclusion bodies in unhemolyzed red cell preparations.

The spectrum of normal red cells showed the two main absorption peaks of oxyhemoglobin at 576 and 542 mμ (Fig. 1). The magnitude of the 542 mμ peak was somewhat higher than the 576 mμ peak and is similar to microphotometrically recorded spectrum of oxyhemoglobin in a localized portion of a single red cell.9 This phenomenon is explained by the baseline being some-
Fig. 3. Absorption spectra from various parts in thalassemic red cell. Upper curve is from inclusion-free area; two lower spectra are from two different inclusion bodies. Note differences in magnitude of the 576-mu and 542-mu peaks inside and outside of inclusion bodies and also between spectrum from normal cell in Fig. 1 and thalassemic cell.

Fig. 4. Absorption spectra from inclusion bodies within thalassemic ghosts. Curves recorded at a 0.01 full scale deflection are three somewhat displaced records of different inclusions showing an absorption band at 535 mµ. Soret band (not shown) had OD around 0.1.
what higher (less than 0.01 optical density units) at 500 m\(\mu\) than at 600 m\(\mu\).

The spectrum of the red cells of both patients outside of an inclusion body showed that the 542 m\(\mu\) absorption peak was in many cases significantly higher than the 576 m\(\mu\) peak, indicating that some of the hemoglobin was in the form of ferrihemoglobin A. A similar spectrum can be obtained by recording a series of spectra ranging from that of a pure solution of oxyhemoglobin A to that of a fully oxidized solution of ferrihemoglobin A.1 The recording of the spectrum of an inclusion body of the same red cell showed the presence of even more ferrihemoglobin but in variable amounts (Fig. 3).

When the spectrum of isolated normal ghosts was recorded, there was no light absorption from 500 to 600 m\(\mu\), indicating that all the hemoglobin had been washed out of the cells. The inclusion bodies within the thalassemic ghosts were of about the same size as seen in the hemoglobin-containing cells. When the spectrum was recorded between 500 and 550 m\(\mu\) at a 0.01 f\(\mu\) scale deflection, a distinct absorption peak of 0.002 optical density units was noted around 535 m\(\mu\) (Fig. 4). The same peak was recorded repeatedly in the same inclusion body, as well as in the other inclusion bodies from different thalassemic red cell ghosts. The 535 m\(\mu\) absorption peak is the main absorption peak of hemichromes in the visual part of the spectrum.1

DISCUSSION

The inclusion bodies in the red cells of patients with \(\beta\)-thalassemia major have been shown to be precipitated \(\alpha\)-chain hemoglobin subunits that are formed in relative excess as a consequence of a defect in the synthesis of \(\beta\)-globin polypeptide chains.10 These inclusions are most prominent in normoblasts11 and appear to be removed from the red cells both prior to their release from the bone marrow and also by the spleen in the peripheral circulation. However, splenectomized patients with \(\beta\)-thalassemia major have a small number of inclusion bodies in peripheral red cells despite an excess of free \(\alpha\)-chains. This appears to indicate that splenic "pitting" is not the only mechanism in the removal of precipitated \(\alpha\)-subunits from the red cells. Intracellular enzymatic proteolysis of excess \(\alpha\)-chains prior to their precipitation could account for part of this phenomenon.12

The following information supports our belief that the inclusions found in the red cells of our two patients were indeed precipitated \(\alpha\)-subunits: free \(\alpha\)-subunits have been demonstrated in the red cells of patients with \(\beta\)-thalassemia;13 peptide maps prepared after tryptic digestion of inclusion bodies in \(\beta\)-thalassemia were characteristic of \(\alpha\)-subunits,10 the formation of hemichromes during incubation is a characteristic of isolated hemoglobin subunits but not of normal hemoglobin tetramers or dimers; and incubation for 24 hr at 37°C failed to induce inclusions in normal red cells.

Microspectrophotometric examination of single inclusions present in the lysed red cells of patients with \(\beta\)-thalassemia revealed only the hemichrome spectrum, while the inclusions of the intact red cells contained both hemichromes and ferrihemoglobin. The ferrihemoglobin probably represents normal hemoglobins A and F that may condense around the denatured \(\alpha\)-hemo-
globin subunits. Consequently, they are not exposed to the normal redox systems of the red cell and undergo progressive oxidation. Ferrihemoglobin, unlike hemichrome, is soluble and was removed from the red cell during preparation of the red cell membranes by repeated washings with a hypotonic buffer.

Both oxyhemoglobin and ferrihemoglobin were present in the thalassemic red cells outside the area of visible inclusions. Only oxyhemoglobin was demonstrable in the incubated control red cells.

The association of unlike subunits in normal hemoglobins appears to prevent hemichrome formation following oxidation of the heme iron, while oxidation of isolated \( \alpha \)- or \( \beta \)-subunits in vitro leads to hemichrome formation and hence to precipitation of the hemoglobin.\(^1\) These events proceeded more rapidly with \( \alpha \)-subunits than with hemoglobin H (Hb \( \beta \)).\(^1\) It is possible that the tetrameric structure of hemoglobin H provides an element of protection against hemichrome formation through subunit interactions and that the reaction is limited by the rate of the dissociation of the molecule into monomeric subunits. Preparations of \( \alpha \)-subunits have been shown to be largely monomeric.\(^13\) While the production of excess \( \beta \)-chains in hemoglobin H disease and excess \( \alpha \)-chains in \( \beta \)-thalassemia major are quantitatively similar,\(^14,15\) substantial accumulation of \( \alpha \)-subunits in the red cells is not a feature of \( \beta \)-thalassemia in contrast to the high proportion (10–40%) of hemoglobin H in the former condition. It is likely that these differences reflect the relatively greater susceptibility of \( \alpha \)-subunits to hemichrome formation, which is a step in the degradation pathway leading to insoluble hemoglobin subunits.

Modell et al.\(^13\) have demonstrated that newly synthesized \( \alpha \)-subunits in the red cells of patients with \( \beta \)-thalassemia are largely dimeric but that these tend, with time, to dissociate into monomers and to disappear from the soluble hemoglobin. It is possible that these dimers are more stable to hemichrome formation and that the effect of incubation in our studies was to facilitate the dissociation of these subunits into monomers, which were then subject to hemichrome formation and consequent precipitation. These incubations were carried out in the absence of exogenous oxidants, and the rate of subunit precipitation may not have exceeded that in circulating red cells. The accumulation of inclusions during incubation, thus, might largely reflect the lack of exposure of the red cells to those mechanisms in the circulation that either "pit" the inclusions from the cells or remove the severely damaged cells.

We postulate that hemichrome formation plays a central role in the precipitation of the excess \( \alpha \)-subunits in \( \beta \)-thalassemia, as has been previously shown in \( \alpha \)-thalassemia (hemoglobin H disease). The irreversible development of an insoluble hemichrome results in progressive oxidation of the excess hemoglobin subunits. The inclusions of precipitated hemichromes produce red cell damage and eventual hemolysis.\(^16,18\)

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