Inclusions of Hemoglobin in Erythroblasts and Erythrocytes of Thalassemia

By PHAEDON FESSAS

FEW EXAMPLES of erythrocytic inclusions due to abnormalities of the hemoglobin molecule are known. The first concerns Hb H.\textsuperscript{1,2} Although the precipitates of Hgb H, such as is observed in the usual test employing brilliant cresyl blue, are produced artificially, there is evidence that some are formed spontaneously in vivo.\textsuperscript{3,4} The second example is that of Hb Zürich; the precipitation of the latter, however, appears to be due to the concomitant influence of potentially toxic substances and may not occur spontaneously.\textsuperscript{5} A further hemoglobin abnormality associated with erythrocytic inclusions was described by Scott et al.,\textsuperscript{6} but the exact connection between abnormal hemoglobin and inclusions has not been clarified. Instability of a hemoglobin fraction has been recently assumed to exist in another case of congenital Heinz-body anemia.\textsuperscript{7}

There are only few reports on the occurrence of Heinz bodies in thalassemia. Two publications deal with the in vitro formation of inclusions upon exposing thalassemic cells to substances known to lead to or enhance the formation of such bodies.\textsuperscript{8,9} It had also been thought at one time that inclusion bodies, such as are produced in Hb H disease, may be formed by brilliant cresyl blue in all thalassemic conditions.\textsuperscript{10} This paper reports preliminary findings on hemoglobinemic inclusions formed spontaneously in vivo in red cells and their precursors, of patients suffering from Mediterranean anemia.

METHODS

Venous blood was examined for routine tests and for inclusions as soon as possible after withdrawal; it was usually collected in the ammonium and potassium oxalate mixture. Bone marrow aspirates were collected in ACD solution. The following methods were applied, alone or in various combinations: 1) Phase contrast microscopy. 2) Vital staining with methyl violet 6B (Merck’s, concentrated) made up to a 1 per cent solution in normal saline. Equal parts of whole blood and stain were mixed in a small tube and were left at room temperature for 10–15 minutes, after which both wet and dry smears were prepared. The latter were used for counting purposes, filing or counterstaining, but in the former the inclusions appear in sharper contrast. This stain was given preference over all other procedures for the detection of the inclusions. 3) Brilliant cresyl blue stain, as used for reticulocyte count. 4) Benzidine stain, according to Lepehne;\textsuperscript{11} 5) Stain for hemosiderin granules. 6) May-Grünwald-Giemsa stain.

RESULTS

a) Description and Staining Properties of Inclusions

The inclusions appear usually as single bodies within normoblasts, reticulo-
Fig. 1.—Blood from case 2, prepared without anticoagulant or staining; phase contrast. Two normoblasts carrying typical large inclusions (lower middle), one without (top edge). Several red cells with single inclusions, one (lower right edge) appearing as a target cell. Two mechanically lysed cells with prominent inclusions are seen in the lower center. Note also vacuolization of several red cells.

cytes or mature red cells. They are round, oval, elongated or irregularly shaped; they are fairly large, usually having a diameter of 1 to 3 \( \mu \) or more. In some orthochromatic normoblasts, their size may be equal to that of the nucleus or even larger; their position in relation to the nucleus varies. Within red cells their occasional central position is reminiscent of target cells. The structures under description are not artifacts due to the staining procedures or the anticoagulants; they can be seen clearly in blood taken with no anticoagulant and examined unstained by phase contrast microscopy. By this latter method the inclusions appear within the cytoplasm of the cells as darker areas of rather loose structure and with serrated outline, as if consisting of more or less densely arranged micelles and not of a homogeneous mass (fig. 1).

Demonstration of the inclusions is best achieved by using vital staining with methyl violet (fig. 2). With this dye the inclusions are very prominent, acquiring a deep purple color; however, their inhomogeneity, as observed by phase contrast, becomes less clear after staining. They can be distinguished
Fig. 2.—Methyl violet stain; three normoblasts and several red cells with inclusions from blood of case 10.

from the nucleus, which stains more palely, having a rather lilac hue (fig. 3). They stain less intensely than the hemoglobinemic inclusions preformed in the erythrocytes of splenectomized patients with Hb H disease.

With brilliant cresyl blue, the inclusions stain a pale homogeneous blue differing only slightly from the blue-green color of the red cell. Since the reticulum stains a darker blue, they can be identified within reticulated red cells; however, the reticulum, especially when abundant, tends to obscure the presence of inclusions.

Fixation with methanol or alcohol abolishes these differential staining properties of the inclusions. Therefore, after counterstaining or by direct staining with May-Grunwald-Giemsa, the inclusions cannot be identified by their coloring properties because they stain like the surrounding hemoglobin. Nevertheless, once observed by the methyl violet stain, they can be recognized on slides stained with May-Grunwald-Giemsa as areas in which the hemoglobin is more densely concentrated, at times in cells having only a thin membrane-like rim of hemoglobin and often surrounded by an unstained hemoglobin-free area. Often the inclusions appear to be the only hemoglobin-containing area of a normoblast, and many schistocytes appear to be formed exclusively by such a dense inclusion body.

The inclusions differ from hemosiderin granules; following the specific staining, one or a few such granules may be found in the neighborhood or on top of the inclusions.

When stained with Lepelne's reagent, considered specific for hemoglobin, the inclusions display the staining properties of hemoglobin and cannot be distinguished from the hemoglobin surrounding them; with this stain the in-
Inclusions appear even less distinct than with the May-Grünwald-Giemsa stain.

In order to establish beyond any doubt the staining properties of the inclusions, dry smears were prepared after vital staining with methyl violet and an informative field was chosen under oil immersion; after removal of the oil with xylol, the slide was subjected to one of the other staining procedures (May-Grünwald-Giemsa, hemosiderin stain, or Lepehne's reagent) and exactly the same field was reexamined. The results confirmed all of the above findings.

In some normoblasts, presumably earlier ones, the material staining with methyl violet does not appear concentrated but is distributed in a fine granular fashion all over the cytoplasm; it may be that the inclusion body is the final stage of aggregation of minute precipitates. Recognition, however, of the stage of maturation of the normoblast when staining with methyl violet is not always easy. In certain instances red cells also appear to contain a fine dust of methyl violet positive granules or a few small, very round and darkly stained inclusions. The relationship of these structures to the large inclusions has not been settled; their connection to the reticulum of the red cell and to Pappenheimer bodies has also to be considered.

b) Other Properties of the Inclusions

Upon hemolysis with distilled water, the inclusions remain within the stroma of the red cell or the normoblast and can be easily found in the sediment upon centrifugation. When hemolyzed in 0.1 per cent Na₂CO₃, considered to effect a more complete freeing of the hemoglobin from the stroma, the inclusions remain within the latter. These hyperresistant, inclusion-carrying cells, together with the normoblasts, when present in large numbers, may be the cause of a slight but not negligible cloudiness in hemolysates used for hemoglobinometry; this can be eliminated by centrifugation.

Upon centrifugation of whole blood, the upper layer of the packed cells contains three to five times more inclusion-carrying cells than the uncentrifuged blood (fig. 4). Whether the lower specific gravity of these cells is due to their younger age or to the presence of the inclusion or to both cannot
be stated at this moment. Interestingly, this is the reverse of what happens to red cells carrying preformed inclusions in splenectomized patients with Hgb H disease.4,12

Whenever the percentage of the inclusion-carrying cells is greatly increased, these latter display a tendency to occur in clusters of varying size. At least this impression is given by observing position and distribution of inclusion-carrying cells in both wet and dry preparations made from methyl violet-stained red cell suspensions. The phenomenon may be due to a certain stickiness of the inclusion-carrying cells (fig. 5).

c) Occurrence of Inclusions

The inclusions have been found in all cases of thalassemia major or intermedia due to homozygosity for two identical or dissimilar β-chain thalassemia genes. They can be readily observed in the circulating normoblasts, mainly at the orthochromatic, pyknotic stage, but also in polychromatophilic cells. Ten to 60 per cent of the circulating normoblasts may present an inclusion. Splenectomy, however, greatly facilitates their detection, owing mainly to the increased number of nucleated red cells in the blood, but possibly also because of some increase in the percentage of inclusion-carrying cells. The inclusions can be readily seen in the more mature red cell precursors of the bone marrow (fig. 6).

In the presence of the spleen, only very few red cells and practically no
Fig. 5.—Occurrence of inclusion-carrying cells in clusters; methyl violet stain, blood from case 8.

reticulocytes are found having typical inclusions. In splenectomized individuals the percentage of inclusion-carrying red cells and reticulocytes is greatly increased: 10 to 30 per cent of the reticulocytes may present this abnormality. Up to 16 per cent inclusion-carrying red cells were counted in one case, but the percentage in mature red cells is usually smaller.

The findings in cases of thalassemia are presented in table 1. Detailed clinical hematologic and biochemical findings are not included; the material, however, displays the greatest variety not only as regards age of patients, severity of anemia, extent of bone changes, degree of splenomegaly, date of splenectomy and transfusion requirements, but also as regards levels of Hb F and A₂. The observations on healthy thalassemia heterozygotes and on the association of thalassemia with other mutant hemoglobin genes will be treated at a later occasion. Because of particular interest, exception is made for a case of Hb Pylos/thalassemia and for a splenectomized case of Hb E/thalassemia disease. The findings in these cases are in all respects similar to those observed in the other cases of thalassemia.

No inclusions of this kind were found in a number of anemias of various etiologies or in other splenectomized patients. Specifically, they were not observed in either marrow or blood in a case of hypochromic, hypersideremic pyridoxine responsive anemia that had been subjected to splenectomy, in three cases of megaloblastic anemia, in iron deficiency anemia and in several other anemias of varying etiology. In a case of homozygous sickle cell disease presenting 30,000 normoblasts per cu.mm., only one normoblast, carrying a rather typical inclusion, was seen in over a thousand examined.

DISCUSSION

Taking into consideration the properties of the intracellular structures de-
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Fig. 6.—Bone marrow preparation from a non-splenectomized patient (case 19), stained vitally with methyl violet. Note normoblasts and red cells, with and without inclusion bodies.

scribed herein, it is concluded that they consist of precipitates of partially denatured hemoglobin. The conclusion that the inclusions consist of hemoglobin is derived from their staining properties: a) acidophilia practically indistinguishable from that of hemoglobin upon staining with May-Grunwald-Giemsa; b) a reaction exactly like hemoglobin with the benzidine stain of Lepehne; c) intense staining with methyl violet in vital preparations, such staining being characteristic of the classical Heinz bodies and of the spontaneously or artificially produced precipitates of Hb H and Hb Zurich. The partial denaturation is the conclusion because: a) intact hemoglobin is soluble while these inclusions are not; b) intact hemoglobin stains vitally only very lightly with methyl violet, but altered hemoglobin stains intensely; c) completely denatured hemoglobin is not expected to give a benzidine reaction indistinguishable from that of intact hemoglobin. This latter point indicates that the heme groups, responsible for the positive benzidine reaction, are present within these inclusions.

Taking further into consideration present-day knowledge and concepts on the disturbance of hemoglobin synthesis in thalassemia,13,14 a number of hypotheses may be advanced at this stage regarding the nature of this precipitated hemoglobin-like material:

a) the inclusions represent aggregates of the excess of α-chains of the hemoglobin molecule which have remained uncombined due to the deficiency in the β-chains and which have not been taken up by γ- or δ-chains;

b) they represent aggregates of abnormal or incomplete β-chains, which, being unstable, precipitate;

c) they represent precipitates of completed hemoglobin molecules which are unstable
Table 1.—Occurrence of Inclusions in Thalassemia

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*n.c. = not counted.
†The number of circulating normoblasts being much lower in this group than in group A, generally fewer cells were examined for inclusions.
‡None observed in at least 200 cells examined.

not because of faulty synthesis but because of a disturbance in the mechanisms normally protecting the hemoglobin molecule from premature breakdown.

Although acceptance or rejection of any or all of the above hypotheses will depend on the precise chemical identification of the substance in question, there is much to be said in favor of the first postulate. Accepting a deficiency of normal β-chains as the primary disturbance in β-chain thalassemias, the presence of an excess of α-chains can be expected. This would be a phenomenon analogous to the excess of β-chains and/or γ-chains in certain α-chain thalassemias. No component which would correspond to free single α-chains or to α-chain polymers has been detected so far in the hemolysates from cases of β-chain thalassemia. Indeed, the lack of such observations, in conjunction with the fact that isolated α-chains can be artificially obtained in vitro, has led to the conclusion that the release of α-chains proceeds forcibly in parallel with the synthesis of β-chains, so that no excess of α-chains results.15,16 If, however, the uncombined α-chains precipitate intracellularly, the inability to detect them in the hemolysate is not surprising. Accordingly, no strict parallelism between synthesis of β-chains on the one hand and α-chains on the other needs to be brought in. The fact that the inclusion phenomenon is so prominent in homozygous thalassemia, where the excess of α-chains can be assumed to be maximal, is also in favor of this postulate. The practically identical findings obtained in the single case of Hb E/thalassemia disease are more difficult to fit into this hypothesis, but are of sufficient interest to be investigated further.

The second hypothesis takes into consideration the theory that an abnormal β-chain, undetectable by the usual technics, is present in thalassemia.13 This hypothesis has not
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received formal proof, but cannot be excluded a priori and the failure to detect a “hidden” β-chain mutation in thalassemia could be explained by assuming that the product of the mutant gene is unstable and precipitates intracellularly. This postulate has the disadvantage that it leaves unanswered the question of the fate of the excess of α-chains.

The third hypothesis can be supported only with difficulty in view of the fact that the inclusion phenomenon is more prominent in the red cell precursors than in the mature red cells; a disturbance in the mechanisms maintaining the integrity of the completed hemoglobin molecule is expected to show its effects rather at the later stages of the life of the erythrocyte. On the other hand, it is not unlikely that in their effort to prevent the hemoglobin from precipitating, the normal enzymatic mechanisms affecting protection of the hemoglobin are unduly taxed. Furthermore, several other—possibly very significant—disturbances may be mediated in both normoblasts and red cells by the mere presence of such large quantities of an apparently useless substance at a very early stage. The latter may not only interfere with the process of hemoglobin synthesis but may also affect the structure and function of the red cell as a whole.

The occurrence of Heinz body-like structures within normoblasts has not been reported so far; a single exception is the case of Schmid et al. It will be of importance to establish whether inclusions occur in all red cell precursors at one stage or another; it is not unlikely that at a given moment we observe a population of normoblasts still carrying the inclusions and another which has extruded them. If the α-chain theory is correct, the inclusion phenomenon may be more prominent in cells unable to synthesize not only β-chains but also a sufficient amount of γ-chains in compensation. Other normoblasts may have lost their inclusion during division, as the inclusion is not expected to divide equally among the daughter cells.

The fact that the percentage of the inclusion-carrying cells decreases sharply after the orthochromatic stage means that the inclusion-carrying cells are removed from the circulation in toto faster than cells not carrying inclusions and/or the inclusions are disposed of while the red cell continues to live. Some schistocytes or cell fragments consisting of methyl violet positive material may represent inclusions which have been extruded together with a thin cytoplasmic envelope. Also, some circulating normoblasts appear as naked nuclei extruded with only a spot of methyl violet positive material attached to them. Conversely, cell fragments carrying no inclusions may represent what has been left over after extrusion of a large inclusion. That the role of the spleen is of primary importance in the process of removal of the inclusions or of their respective cells is obvious from the striking rise in the percentage of abnormal cells after splenectomy; evidently the spleen masks the inclusion phenomenon, the true dimensions of which can be observed in the blood only following removal of this organ. The analogy to what has been observed and proven in the case of siderocytes is obvious. However, since even after splenectomy the percentage of the mature cells carrying inclusions is low, it can be concluded that the inclusions, or the inclusion-carrying cells, can be disposed of in the absence of the spleen. The elucidation of the mechanism of removal of these cells and/or their inclusions will depend on further studies including cross-transfusion experiments with labeled erythrocytes, the inclusions serving as a second tag. The apparent stickiness of the inclusion-
carrying cells, a phenomenon which may or may not be related to relevant observations on reticulocytes,19 may also play a role in the rapid rate of removal of these cells from the circulation. In addition, it may be necessary to consider not only extrusion but also intracellular degradation of the precipitate.

In view of the great extent of the inclusion phenomenon in thalassemic normoblasts, it can be assumed that the inclusions, alone or together with the cells carrying them, contribute largely to the observed early rise in the excretion of radioactively labeled stercobilin.20 In analogy, they may be expected to contribute to the secondary rise of radioactivity over the spleen when using the appropriate labels, thus presenting as a population of very short-lived red cells.21,22 In a certain sense the inclusion phenomenon offers strong cytologic evidence for the "ineffective" erythropoiesis occurring in thalassemia.20,22-24 The formation of intracellular precipitates during early stages of hemoglobin synthesis may have to be taken into consideration not only for the in vivo studies but also for systems employing intact cells for the study of hemoglobin synthesis or iron metabolism in thalassemia in vitro.

It is appropriate to refer here to certain previously reported cytologic abnormalities of thalassemic cells. The granulations described first by Astaldi et al.25 have an entirely different size and distribution over the cytoplasm of the normoblast, besides being periodic-acid-Schiff positive. Silverstroni et al.10 reported inclusions staining with brilliant cresyl blue in the red cells of a few splenectomized cases of "constitutional microcytic anemia"; these are most probably identical to the inclusions described here. The inclusions mentioned by Josephson et al.8 as occurring spontaneously in a small number of patients with various forms of thalassemia may or may not be identical, as no details are given by these authors.

As regards the findings revealed by electron microscopy, the high density particles26-31 can hardly be compared to the inclusions described here because of their size, number and distribution; apparently they represent ferritin molecules or aggregations thereof. However, it is possible that some gross particles,27 found in the stroma, are identical to the methyl violet positive inclusions; some of the preparations belonged to splenectomized cases. Similarly, certain structures observed by Bessis et al.28,29 in thalassemic red cells and normoblasts, and referred to as "substance amorphe," are very likely identical to the inclusions described here because of their size, form and location; the authors, whilst admitting that practically nothing could be said about the nature of these structures, considered the possibility that this material is apoferritin.29,30 In their previous paper, however, Bessis and Breton-Gorius favored the view that these polycyclic or angular masses represented denatured hemoglobin or one of its constituents.28

Since this is a preliminary report, of necessity both the presentation of the findings and their discussion have not covered all aspects of this cytologic abnormality and of its relationship to the hematologic, biochemical or other cytologic disturbances occurring in thalassemia. The problems arising through the observations presented here can be approached from several angles; investigation of some aspects is in progress in this laboratory.
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SUMMARY

Large, usually single inclusions, having the staining properties of Heinz-bodies and occurring spontaneously in thalassemic cells, are described. Their frequency is greatest in the normoblasts, either circulating ones or those found in the bone marrow, and decreases sharply in more mature red cells. Splenectomy unmasks this inclusion phenomenon to a large extent. Some staining and other properties of these inclusion bodies are described.

It is concluded that these inclusions represent precipitated hemoglobin, very likely uncombined α-chains. The findings are discussed and related to present-day knowledge on hemoglobin synthesis and erythrocyte turnover in thalassemia.

SUMMARIO IN INTERLINGUA

Es describite grande, usualmente singulari inclusiones, con le proprietates tincturatori de corpores de Heinz, que occurreva spontaneamente in cellulatas thalassemic. Lor frequentia es le plus alte in normoblastos, tanto in illos in le circulation como etiam in illos trovate in le medulla ossee, sed ille frequentia declina acutemente in erythrocytos plus matur. Splenectomia demasca iste phenomeno de inclusion in grande mesura. Certe proprietates tincturatori e alte re que characterisa iste corpores de inclusion es describite.

Es concludite que iste inclusiones representa precipitate hemoglobina, probabilissimemente non-combine catenas α. Le constatationes es discutite e relationate con nostre presente cognoscentias in re le synthese de hemoglobina e le ciclo vital de erythrocytos in thalassemia.

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REFERENCES

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12. Unpublished data from this laboratory.

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