Intraerythrocytic Crystals in a White Patient with Hemoglobin C in the Absence of Other Types of Hemoglobin

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Crystals of hemoglobin were observed in the erythrocytes of a white boy, whose hemoglobin was of the “pure” C type. Electrophoretic analysis of hemolysates of four other members of his family revealed hemoglobins A and C. A description of the anomalous intraerythrocytic structures, together with hematological and electrophoretic studies of this patient and his family, is warranted because spontaneously appearing crystals of the type observed have not been reported previously, because of the rarity of “pure” hemoglobin C disease, and because hemoglobin C has not been described heretofore in members of the white race.

Case Report

Charles M., a white male of Italian parentage, was mildly anemic in early childhood and was treated by his physicians with various antianemic drugs, including iron and liver extract. The mother thinks that the patient’s “color was not as good” as that of the other children and that there has been considerable variation in the degree of pallor at different times. Jaundice had not been noted. The patient played and worked vigorously and participated and excelled in competitive sports.

At the age of fourteen he began to have intermittent abdominal pains, which persisted for several weeks, followed by nausea, vomiting, abdominal distention and diarrhea. He was referred to the Archer Clinic in Greenville, Mississippi, with the admitting diagnosis of “possible intestinal obstruction.” There was no elevation of temperature. The blood pressure was 120/60. A mass in the left upper quadrant extended to the umbilicus. A flat plate of the abdomen showed a normal gastrointestinal pattern and an enlarged spleen. Intravenous urograms and urine examinations revealed no evidence of genito-urinary abnormality. Rectal examination was negative and there was no blood in the feces. The hemoglobin was reported to be 80 per cent, RBC 4,330,000 per mm.³, WBC 7,600 per mm.³ and the differential 79 per cent neutrophils, 19 per cent lymphocytes and 2 per cent monocytes.

Exploratory laparotomy was performed and a 2.3 kilogram spleen measuring 200 by 140 by 100 mm. was removed. The spleen showed no gross abnormality other than diffuse engorgement with blood. Microscopic examination revealed a thin capsule without adhesions. The trabecular pattern was normal. The lymphatic nodules were smaller than normal, were widely separated, and had ill-defined germinal centers. The red pulp contained many erythrocytes, an increased amount of fibrous tissue and relatively few white cells. The venous sinusoids were dilated and many were filled with red cells. The endothelial cells of the vascular channels were conspicuous. Pigment-laden macrophages were demonstrable in the splenic pulp. In some of the trabecular arteries and in occasional pulp sinusoids, there were...
small aggregates of red cells which seemed to be fused together in reddish homogeneous masses intermingled with the well-defined and separate erythrocytes.

Sickled cells, pooling of blood at the margins of Malphighian bodies and siderofibrotic nodules characteristic of sickle cell disease, and islands of extramedullary hematopoiesis and foam cells of the type seen in thalassemia were not demonstrable.

The postoperative course was uneventful. Hematological studies performed on five occasions during the next two years revealed a persistent low grade leukocytosis, a normal white cell distribution, moderate poikilocytosis and anisocytosis with macrocytes and nucleated red cells. The hemoglobin ranged from 14.1 to 16 Gm. per 100 ml., and the red cell counts from 4,580,000 to 4,830,000 per mm.3.

The patient was first seen in consultation at the Hematology Laboratory of the University of Tennessee at the age of 16, eighteen months after splenectomy. He was an alert young man who appeared to be in good health. He was well nourished and had good muscular development. With the exception of a slight pallor of the mucous membranes and a questionable icteric tint, there were no physical abnormalities.

The hemoglobin was 14.2 Gm. per 100 ml., RBC 5,180,000 per mm.3 and hematocrit 37 ml. per 100 ml., MCV 71 cubic microns, MCH 27 micromicrograms and MCHC 38 per cent. Blood studies on two other occasions within a three month period revealed no significant changes in erythrocyte values. The reticulocytes varied from 88 to 94 per cent in brilliant cresyl blue wet preparations. The amount of granulofilamentous substance within many of the erythrocytes was minimal.

The 1 minute (direct) serum bilirubin was 0.2 mg. with a total bilirubin of 1.6 mg. per 100 ml. Microscopic examination of the urine and qualitative tests for bilirubin, hemosiderin, albumin and glucose were negative. The urine urobilinogen concentration was 1.9 Ehrlich units per 100 ml.

Fragility tests revealed a striking increase in erythrocyte resistance to hypotonic saline solution. Hemolysis began at 0.40 per cent NaCl and was not complete in distilled water (control 0.46 per cent to 0.36 per cent NaCl). Red cells were grossly demonstrable in the sediment in dilutions down to and including 0.12 per cent NaCl.

The WBC varied from 12,750 to 15,000 per mm.3 with an essentially normal cell distribution, except for a slight absolute increase in lymphocytes and monocytes. Thrombocytes were normal.

The sedimentation rate by the Wintrobe method was 2 mm. per hour. Drops of whole blood sealed under a coverslip and whole blood mixed with sodium metabisulfite were negative for sickling after 30 minutes and also after 24 and 48 hours. The blood group was A, D positive.

Abnormalities observed in the smears of peripheral blood included moderate anisocytosis and poikilocytosis, and numerous hypochromic cells. Target cells varied from 10 to 30 per cent. Many of the erythrocytes had surface irregularities and folds (fig. 1). Diffusely basophilic cells were conspicuous, but stippled cells were hard to find. Many of the erythrocytes had varying numbers of very small refractile bodies. These bodies were as readily demonstrable in unstained as in stained preparations, and gave a negative prussian blue reaction for free iron. There were 7 to 18 metarubricytes per 100 WBC. The most striking abnormality was the presence of crystals in about 2 per cent of the mature erythrocytes (figs. 1, 2, 6).

Smears of sternal bone marrow revealed numerous nucleated cells and a leukocyte to nucleated red cell ratio of 1:1.3. There was a slight relative and absolute increase in the immature erythrocytic cells, but the majority of cells were rubricytes and metarubricytes of normal size and color. Megakaryocytes were readily demonstrable and the thrombocytes numerous. The distribution of leukocytes was within normal limits. Section of the marrow clot revealed a fat to cell ratio of approximately 1:4. The cellular and trabecular pattern was normal. Pigment-laden macrophages were conspicuous.

**INTRAERYTHROCYTIC CRYSTALS**

Unstained moist preparations of whole blood taken directly from a finger puncture, moist preparations of oxalated blood and red cells removed from the clot, whole blood mixed with sodium metabisulfite and marrow aspirated from
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the sternum all revealed crystals in about 2 per cent of the mature erythrocytes (figs. 1, 2, 3, 6). On microscopic examination of dried blood smears and of thin moist preparations, these structures appeared as dark, elongate, six-sided objects with blunt or pointed ends. In moist preparations of blood diluted with 0.85 per cent NaCl, it was possible to rotate the crystals and to see them from
Fig. 2.—Drawing showing hemoglobin crystals
A. Brilliant cresyl blue moist preparation
B. Blood smear, Wright’s stain.

various angles. When seen from the end, with the line of vision parallel to the long axis, they had four sides of approximately equal length, arranged at right angles to each other. Since the three axes were at right angles to each other and the two lateral axes equal, the crystals belong to the tetragonal system. There was variation in the size and number of crystals within various cells (fig. 2), but the majority consisted of single crystalline structures, 6 to 10 micra long and 2 to 3 micra wide. The long sides of the majority of the crystals were straight and parallel, but some were bent in the middle and had slight central constrictions suggesting conjoined crystals.

In the dried blood smears, many of the crystals appeared to be free. In others, the cell membrane was draped around the crystals, the margins appearing as a thin encircling line. In moist preparations, the crystals appeared to lie within
Intraerythrocytic crystals in C type of hemoglobin

Fig. 3.—Intraerythrocytic crystals. Moist preparation of whole blood. Phase microscope.

the red cell envelopes like brown angular blocks in cellophane bags (fig. 2). The crystals had the same color as the hemoglobin in the other cells but were of darker hue. In most of the erythrocytes containing the crystals, all of the hemoglobin appeared to be concentrated within the crystal structure, leaving the rest of the cell devoid of color. In a few cells there were varying amounts of residual hemoglobin which had not assumed the crystalline form.

Exposure of whole blood to carbon monoxide, producing carbonmonox-hemoglobin, and addition of sodium nitrite, resulting in methemoglobin, did not appear to alter the percentage of red cells containing crystals. On the other hand, exposure of whole blood to hydrogen sulfide gas, producing sulfhemoglobin, seemed to reduce the number of crystals significantly.

Dried blood smears or moist preparation of whole blood were stained with a number of different stains in an attempt to identify the compound forming the crystals. The crystals did not stain with sudan IV and, therefore, were not neutral fat. Stains for nuclear material, namely brilliant cresyl blue, stilbamidine, berberine, auromine, rhodamine, and phloxine were not taken up by the crystals. Marrow tissue fixed with formalin and stained with hematoxylin and eosin revealed the crystals in about 2 per cent of the mature erythrocytes.

Moist preparations of whole blood stained with brilliant cresyl blue revealed the crystals as chartreuse objects. This effect was thought to represent a combination of the blue background of the stain with the yellowish color of the more concentrated hemoglobin in the crystals. A few of the cells containing crystals also contained blue, reticular material lying next to the unstained crystal structure, which indicated that some of the crystals were present in immature erythrocytes.

The number of crystal structures did not appreciably increase on standing in sealed moist preparations for 24 hours, but they became less numerous after standing for 1 week. Shaking of whole, oxalated blood with glass beads for 6
hours caused a significant decrease in the percentage of red cells containing crystals. The degree of hemolysis, however, was no greater than that of normal blood exposed to the same conditions. The crystals were observed in hypotonic solutions of sodium chloride, but could not be found after 30 minutes in a 2 to 1 mixture of distilled water and whole blood.

Dried, unfixed blood smears were prepared on quartz slides and spectra of individual red blood cells were obtained in the visible and ultraviolet light range (6000–2300 Å) by a method essentially similar to the one described by Metcalf. Reflecting quartz optics, a Gaertner spectroscope and a carbon arc were used for the ultraviolet range. Photographs of the entire spectrum were taken. Densitometric readings were determined under a microscope by means of a photoelectric cell. In this way the blank, red cell and crystal spectra could be compared at each wavelength examined. In this range the spectra of red cells containing crystals were found to be identical to those of red cells without crystals. The Soret band was clearly identified. In addition, absorption bands were found at 2450 Å, 2800 Å and 3500 Å. No absorption maxima could be clearly demonstrated above 5000 Å. The extremely thin hemoglobin layer in individual red cells was thought to be responsible for this failure. These spectra compare well with the hemoglobin spectra reported by Jope and Drabkin and Austin.

Family Survey

The mother and father of the patient are Italians. They were in good health at the time of the examination and had no history of severe illnesses or chronic disability. The mother has eight siblings and the father six, none of whom are known to have anemia, jaundice or splenomegaly. One brother (Gerald M) and one sister (Rose M) of the patient are in good health. Hematological studies of the father, one maternal uncle (Dominic D) and Gerald and Rose revealed normal hemoglobin, erythrocyte and serum bilirubin values and normal reticulocyte percentages. Similar findings were observed in the mother, with the exception of a mild anemia of the hypochromic type, which responded promptly to iron therapy. Sickling was not demonstrable in any of these individuals. Target cells in the blood of the brother (Gerald M), the mother, the father and maternal uncle (Dominic D) constituted 1.5, 0.4, 1.3 and 0.2 per cent of the erythrocytes respectively. There were no demonstrable target cells in the blood smears of Rose M. Stippled cells were not increased in any members of the family examined.

Electrophoretic and Chemical Studies

Methods

The hemoglobin solutions were prepared according to the method of Itano. Paper electrophoresis was performed according to Kunkel and Tiselius utilizing a veronal buffer, ionic strength 0.06 M, pH 8.8. Macroelectrophoretic analysis was carried out in the Klett-Longsworth modification of the Tiselius apparatus with cacodylate buffer, ionic strength 0.1 M, pH 6.5 and also with veronal buffer, ionic strength 0.06, pH 8.8. Fetal hemoglobin was identified by means of a modified alkali denaturization method worked out in this laboratory.
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Results

The electrophoretic mobility of Charles M.'s hemoglobin, as determined by paper electrophoresis (fig. 4) and by the Klett-Tiselius apparatus, revealed only hemoglobin C. The identity of hemoglobin C was verified by electrophoretic analysis of mixtures of his hemoglobin with that of patients having known sickle cell-hemoglobin C disease (S-C). No fetal hemoglobin could be identified. Gelling was not observed when a 30.9 Gm. per 100 ml. solution of Charles M.'s hemoglobin was exposed to a CO₂ atmosphere in a shaker for one hour.

The patient's sister, Rose M., had only hemoglobin A. No fetal hemoglobin could be demonstrated.

The hemolysates of both parents, the brother, Gerald M., and the maternal uncle, Dominic D., revealed two hemoglobin components when examined by paper electrophoresis (fig. 4). These two components were found also in the Klett-Tiselius apparatus when a cacodylate buffer, ionic strength 0.1 M, pH 6.5 was used (fig. 5 A). The leading component had the same mobility as the hemoglobin C of Charles M. The second component was identified as hemoglobin A. A quantitative estimation of the hemoglobins from the electrophoretic patterns revealed the following values: father 60 per cent A and 40 per cent C; mother 62 per cent A and 38 per cent C; Gerald M. 57 per cent A and 43 per cent C.

When, however, veronal buffer, ionic strength 0.06 M, pH 8.8 was used for electrophoresis in the Klett-Tiselius apparatus, a third component lying between
Fig. 5.—Ascending electrophoretic pattern (Klett-Tiselius) of the father
A. Caedvylate buffer, 0.1 M, pH 6.5.
B. Veronal buffer, 0.06 M, pH 8.8, showing third component between A and C, thought to be red cell stroma.
hemoglobins A and C could be demonstrated (fig. 5B). This component had an electrophoretic mobility similar to hemoglobins S or D and was initially interpreted as consisting of one of these hemoglobins. Further experiments indicated that this third component was apparently due to an impurity of the hemoglobin solutions, probably red cell stroma.

**Discussion**

The clinical and hematologic features of our patient with "pure" hemoglobin C disease are similar to those reported by others. These individuals have a hemolytic anemia of mild degree with normal or slightly increased serum bilirubin, increased urobinogen excretion and erythrocytic hyperplasia of the bone marrow. In all cases the spleen is enlarged. The number of target cells is increased and osmotic fragility tests show increased resistance of the red cells to hypotonic saline solutions. Reticulocytes tend to be increased and nucleated red cells are demonstrable in the peripheral blood of some of the patients. Their red cells have a decreased survival time when transfused into normal recipients.

The unusual features of our case are the occurrence of "pure" hemoglobin C in a white person, the crystals of hemoglobin, the refractile bodies in the erythrocytes, and the abnormally high reticulocyte counts. It is probable that the high reticulocyte count is in part accounted for by the refractile bodies. Iron stains exclude the possibility that these structures are siderotic granules.

The spleen has not been examined in a sufficient number of patients with
"pure" hemoglobin C disease to warrant final conclusions about the characteristic anatomic findings, but tissue changes in the case reported by Singer et al.\(^2\) and in our patient were the same, consisting of congestive enlargement, dilated sinusoids, increased prominence of endothelial cells, and maintenance of splenic architecture without siderofibrotic nodules, foam cells, or abnormal pigmentation.

The relationship of the splenectomy to the formation of intraerythrocytic crystals is conjectural. Blood smears made previous to splenectomy were not available for examination. Failure of the technologist to see and report such crystals at the time of splenectomy cannot be taken as proof of their absence. Similar crystals have never been described following splenectomy in normal individuals, nor in individuals with hemolytic anemia, including the patient with pure hemoglobin C disease described by Singer and associates.\(^2\)

The acute abdominal pains, which characterized one episode in the history of our patient and which led him to have an exploratory laparotomy, have been observed in other patients with "pure" hemoglobin C disease and are similar to the episodes of abdominal pain observed in spherocytic hemolytic anemia and sickle cell anemia.

The crystalline structures observed are considered to be crystals of hemoglobin or a hemoglobin derivative for the following reasons. The red cells containing crystals have little or no residual hemoglobin outside of the crystalline structure. The crystals appear in fresh, unstained preparations and do not appear to be artefacts due to the preparation of films or addition of anticoagulants. The color of unstained crystals is the same as that of the hemoglobin in the neighboring red cells. The crystals stain the same as hemoglobin. The visible and ultraviolet spectrum of single crystals and of individual red cells without crystals is identical. The crystals, like the hemoglobin crystals described by Drabkin\(^19\) and others,\(^2\) belong to the tetragonal system.

Since the family under study is of Italian origin, the possibility must be considered that thalassemia minor is a factor responsible for the hemolytic anemia of Charles M. He has numerous target cells. The MCV and MCH are low, the MCHC normal (microcytic erythrocytosis), and his red cells show an increased resistance to hypotonic saline solutions. While these findings are suggestive of thalassemia, they are also seen in sickle cell-hemoglobin C disease and hemoglobin C trait.\(^2\)\(^1\)\(^2\) In our judgement, Charles M. does not suffer from thalassemia. His red cells do not show any increase in stippling. Neither of his parents nor other members of his family who were examined have a history of chronic, refractory, hypochromic anemia, and none, with the exception of the mother, were anemic at the time of examination. Her hypochromic, microcytic anemia responded rapidly to iron therapy. None of the relatives have an increase in target or stippled cells, or splenomegaly.

**Summary**

Tetragonal crystals of hemoglobin were observed in approximately 2 per cent of erythrocytes of a sixteen-year-old white male of Italian parentage, who had hemoglobin C in the absence of A, S, and F.
The clinical and hematological features of the patient described are similar to those with "pure" hemoglobin C disease reported by others. The essential features are: chronic anemia of a mild degree, congestive splenomegaly, evidence of increased red cell destruction, hyperplastic marrow with increased numbers of immature erythrocytes in the peripheral blood, target cells and increased erythrocyte resistance to hypotonic saline solutions.

Electrophoretic analysis of hemolysates of both parents, one brother and a maternal uncle revealed hemoglobin A and C. The hemolysate of one sister revealed only hemoglobin A. None of the relatives examined had hemoglobin S or F and none showed hematologic abnormalities.

Addendum

Re-examination of Charles M. twenty-seven months after splenectomy revealed that he had gained weight and become more muscular. Otherwise the physical examination remained essentially unchanged. He was then the star pitcher of his high school baseball team. The peripheral blood showed the following findings: hemoglobin 15.2 Gm. per 100 ml., RBC 4,850,000 per mm.³ WBC 11,100 per mm.³, reticulocytes 94 per cent, differential: 23.5 per cent neutrophils, 5.0 per cent eosinophils, 1 per cent basophils, 66.5 per cent lymphocytes and 3.5 per cent monocytes. The smear again showed moderate anisocytosis and poikilocytosis of the red blood cells with 21 per cent target cells and numerous diffusely basophilic cells. Twenty-four nucleated red blood cells were found per 100 WBC. Approximately 2 per cent of the red blood cells contained the crystal-like structures described above. The 24 hour urine urobilinogen was 36.0 Ehrlich units and the 24 hour fecal urobilinogen 1,339 Ehrlich units.

Dr. Eric Ponder graciously consented to examine a specimen of the peripheral blood. He writes as follows: "These (the crystal-like structures) are easily seen under the ordinary microscope in fresh preparations and also by phase contrast, where they appear as little rod-like bodies, clearly surrounded by what looks like the remains of the red cell surface ultrastructure. Under the polarizing microscope the rod-shaped bodies do not show enough birefringence to be detected by a λ/30 compensator. If any birefringence is present, it is much less than seen in sickled cells and very much less than that of hemoglobin crystals prepared from solutions of hemoglobin."

In addition, the following experiment was performed: Five ml. of whole blood was collected in 3 per cent citrate. The red blood cells were washed three times with 3 per cent citrate and resuspended in 25 ml. of 3 per cent citrate. This suspension was kept in the refrigerator at 6°C for 4 weeks. Under these conditions the relative number of cells containing the crystal-like structures appeared to increase in contrast to whole blood of the patient kept at refrigerator temperature where the number of crystal-like structures decreased markedly after storage for one week. When a small drop of the citrate-red cell suspension was placed on a slide, covered with a coverslip and allowed to dry partially, a large number of hemoglobin crystals formed between the remaining red blood cells. On further drying, the red cells were destroyed, leaving numerous well-defined buff-colored crystals. When completely dry, the crystals cracked and became less refractile.
Crystallos tetragon de hemoglobina esseva observate in approximativemente duo pro cento del erythrocytos de un patiente mascule de 16 annos de etate, de parentage italian, con hemoglobina C e nulle hemoglobimia A, e F.

Le caracteristicas clinic e hematologic del patiente esseva simile al characteristics de patientes con morbo de “pur” hemoglobina C secundo le reportos de altere autores. Le characteristicas essential esseva: anemia chronic in leve grado, splenomegalia congestive, signos de augmentate destruction de erythrocytos, medulla hyperplastic con augmentate numeros de erytyrocytos immatur in le sanguine peripheric, cellulas corohliforme, e augmentate resistencia erytrocytie a solutiones salimi hypotomiic.

Le analyse electrophoretic de hemolysatos ab patre, matre, un fratre, e un omicle materne revelava hemoglobina A e C. Le hemolysato de un soror revelava solo hemoglobina A. Nullo del parentes examinate habeva hemoglobina S o F, e nullo monstrava anormalitates hematologic.

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