Hemoglobin Pyrgos $\alpha_2\beta_2^{83\,(EF7)\,Gly \rightarrow Asp}$: A New Hemoglobin Variant in Double Heterozygosity With Hemoglobin S

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An electrophoretically fast-moving hemoglobin variant was found to be present, together with hemoglobin S, in the hemolysate of the erythrocytes of a 3-yr-old Greek boy. Electrophoresis of the parents' erythrocyte hemolysates revealed that the father was an AS heterozygote, while the mother was a carrier of the variant hemoglobin. A sibling was also found to be a carrier. The amount of the mutant hemoglobin in the peripheral blood of the propositus, his mother, and his brother was 62.2%, 52.5%, and 51.1%, respectively, as determined by column chromatography. The patient's peripheral blood smear showed mild anisocytosis, microcytosis, and hypochromia. Similar but less pronounced red cell abnormalities were found in the other two carriers. Structural analysis of the variant hemoglobin revealed substitution of an aspartic acid for the glycine residue at the β^{83} (EF7) position. This new hemoglobin was named hemoglobin Pyrgos. All the carriers of hemoglobin Pyrgos are clinically healthy, and there seems to be no interaction between hemoglobin Pyrgos and hemoglobin S as manifested clinically.

HEMOGLOBIN VARIANTS are of great interest because they help us to understand better the structure and function of the normal and abnormal hemoglobin molecule. In this report, we present the clinical and laboratory findings of a 3-yr-old Greek boy and members of his family, carriers of a new hemoglobin variant, and the structural analysis of the variant. The new mutant hemoglobin has been named hemoglobin Pyrgos after the Greek town from which the proband's mother, also a carrier, originates.

MATERIALS AND METHODS

Hematologic determinations were performed by standard methods.[2] Hemoglobin electrophoresis was carried out on starch gel at pH 8.6.[3] Hemoglobin thermal stability[2] was done on 3-day old blood samples which were shipped on ice from Athens to New York. A control blood sample from a normal person drawn at the same time was shipped in the same container. Red cell inclusion bodies were sought by incubating at 37°C two parts of whole blood with one part of 1% brilliant cresyl blue in 0.9% NaCl.

Hemolysates were prepared by the method of Drabkin.[4] Fetal hemoglobin was determined by the 1-min alkali denaturation method.[5] The percentage of hemoglobin A_2 was determined by diethylaminoethyl-cellulose (DEAE) column chromatography as described below. Sickle cell preparations were done by the sodium metabisulfite method.[2]

Chromatographic analysis of the various hemoglobin fractions of the propositus and his father's hemolysate was done using DEAE-cellulose (DE 52, Whatman Inc., Clifton, N.J.) equilibrated with 10 m*M* Tris NaH_2PO_4 buffer at pH 8.5. Forty to sixty milligrams of hemoglobin in solu-

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Table 1. Hematologic and Hemoglobin Values in Family With Hb Pyrgos

Hematologic Values	Propositus	Brother	Mother	Father
Hemoglobin concentration (g/dl)	12.6	12.3	12.5	15.8
RBC (10^{12}/liter)	5.74	5.08	4.83	4.98
PCV (%)	40	39	41	50
MCV (fl)	69.6	76.7	84.8	100.4
MCH (pg)	21.9	24.2	25.8	31.7
MCHC (g/dl)	31.5	31.5	30.4	31.6
WBC (10^9/liter)	10.2	13.5	7.0	8.4
Reticulocytes (%)	1.3	1.1	1.1	0.6
Red cell morphology				
Anisocytosis	++	+	+	−
Microcytosis	++	+	+	−
Hypochromia	++	+	+	−
Serum iron (μg/100 ml)	133	66	96	116
Hemoglobin composition				
Hb Pyrgos	62.2	51.1	52.5	−
HbA	−	46.5	44.6	59.7
HbS	35.2	−	−	37.6
HbA_2	2.6	2.4	2.9	2.7
HbF (alkali-resistant Hb)	0.7	1.3	0.5	0.6

tion were applied on a 1 × 25-cm column, and the various fractions were eluted with a pH gradient from 8.5 to 7.4 of 300-ml total volume at room temperature. The hemoglobin fractions of the mother and siblings' hemolysates were separated using carboxymethyl-cellulose (CM 52, Whatman, Inc., Clifton, N.J.) equilibrated with 10 m*M* NaH_2PO_4–Na_2HPO_4 buffer at pH 6.5. Forty to sixty milligrams of hemoglobin in solution were applied on a 1 × 25-cm column, and the various fractions were eluted with a pH gradient from 6.5 to 7.4 of a 300-ml total volume at room temperature. The optical densities were read at 415 nm with a Beckman DB-G spectrophotometer.

For structural analysis, the variant hemoglobin was purified, on a preparative scale, by CM-cellulose column chromatography as described above.

Globin preparation, globin-chain separation by urea-CM-cellulose column chromatography, S-aminoethylation, tryptic digestion, peptide high voltage electrophoresis, and chromatography procedures were carried out according to Clegg et al.[6] with some minor modifications. The variant peptide was eluted from a preparative chromatogram with 6 *N* hydrochloric acid (constant boiling) into a 100 μl capillary tube which then was sealed. After hydrolysis for 18 hr at 108°C, the capillary tube was emptied and the contents subjected to amino acid analysis with a Jeolco Model JLC-6AH amino acid analyzer.

N-terminal amino acid identification of the variant peptide was done by the densylation procedure described by Gray.[7]

All the chemicals used were of analytical grade except urea which was deionized with a mixed bed ion exchange resin (Rexyn I-300 (H-OH), Fisher Scientific Co., Fair Lawn, N.J.) before use.

CASE REPORT

A 3-yr-old Greek boy was seen for a febrile illness of several days' duration. On physical examination, the patient had mild generalized lymphadenopathy, and the spleen was palpable 1.5 cm below the left costal margin. The hemoglobin was 12.2 g/100 ml. The leukocyte count was 9900/cu mm of which 61% were lymphocytes. Many of the lymphocytes were atypical. There was mild anisocytosis, microcytosis, and hypochromia of the red blood cells. The hematologic values of the patient and other members of his family are shown in Table 1.

Peripheral blood smears of the proband's mother and 1-yr-old brother showed similar but less pronounced red blood cell abnormalities. The father's blood count, and peripheral blood smear were normal.

The febrile illness was diagnosed as infectious mononucleosis. The fever subsided within a few days, and the spleen was no longer palpable 1 mo later.

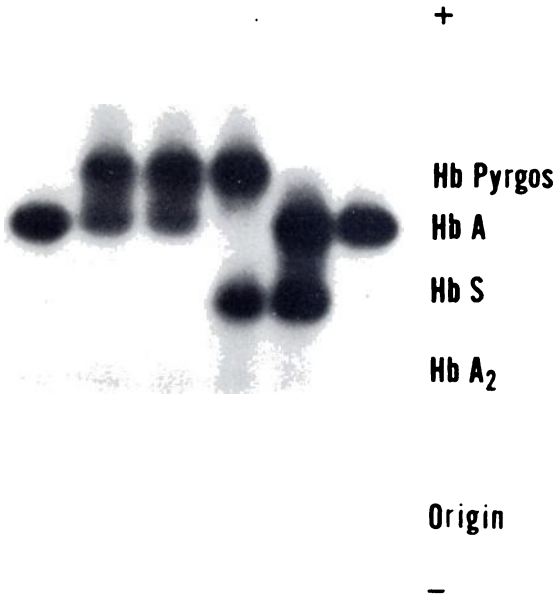

Fig. 1. Vertical starch gel electrophoresis of red cell hemolysates from the members of the family with Hb Pyrgos. Tris–EDTA–boric acid buffer, pH 8.6.[3] Benzidine stain. From left to right: normal control, brother, mother, propositus, father, normal control.

RESULTS

Hemoglobin Studies

Starch gel electrophoresis of the hemolysate of the propositus showed a major fast-moving component, as well as a minor component having the electrophoretic mobility of hemoglobin S (Fig. 1). The father's hemolysate showed an AS pattern. The hemolysates of the mother and brother showed that both were carriers of hemoglobin A and the fast-moving variant. Sickle cell preparations were positive for the propositus and his father but negative for his mother and brother.

The heat stability test showed no heat unstable hemoglobin in the hemolysates of the propositus or any member of his family. No inclusions were seen in the red cells of the various members of the family after incubation at 37°C for up to 4 hr with brilliant cresyl blue dye.

The various hemoglobin fractions in the hemolysate of the four family members, as they were calculated from the chromatographic separations, are shown in Table 1, together with the fetal hemoglobin values as measured by alkali denaturation.

Structural Studies

When the proband's whole hemolysate globin was separated by urea CM-cellulose chromatography, it became evident that the variant chain was a β chain because it was eluted before the β^A chain under standardized chromatographic conditions.

Fingerprints of the tryptic digests of the S-amino-ethylated β chains of the purified hemoglobin variant showed that the tryptic peptide 10 was missing from its normal position and a new peptide with slower mobility toward the

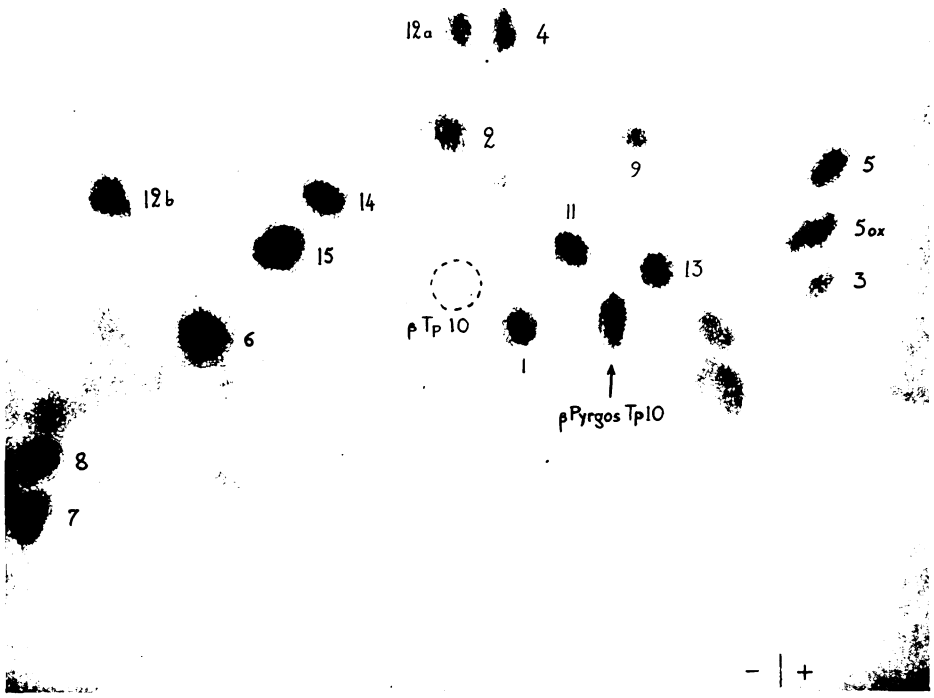

Fig. 2. Map of ninhydrin-developed peptides prepared from tryptic digestion of S-aminoethylated β^{Pyrgos} globin chain. Paper electrophoresis (from right to left) at pH 4.7 was followed by descending paper chromatography (lower to upper) according to Clegg et al.[5] Line of application is at the right lower corner. Numbers refer to tryptic peptides. In β^{Pyrgos}, tryptic peptide 10 is absent (open circle) from its usual position and shifted to a new position (arrow).

cathode was apparent (Fig. 2). This finding was confirmed by staining the fingerprints with specific stains for histidine and sulfur-containing amino acids.[8]

The amino acid composition of the variant peptide is shown in Table 2 and indicates that a glycine residue normally occupying the 83rd position in the β chain has been replaced by an asparagine or an aspartic acid. Taking into consideration the charge difference between hemoglobin A and the hemoglobin variant, and the genetic code, we concluded that the amino acid substitution was an aspartic acid. This conclusion was verified by the N-terminal analysis which showed that the densylated amino acid at the N-terminal of the tryptic peptide 10 of the hemoglobin variant was an aspartic acid and not a glycine.

DISCUSSION

Hemoglobin Pyrgos has a substitution of a charged amino acid for a neutral one at position β^{83}, or β EF7 according to Perutz's helical notation. This residue is located on the external surface of the hemoglobin molecule and is not expected to affect its stability.[9] This expectation is in accord with the findings of normal thermal stability of the hemolysates of the carriers, and the negative results of the incubation of their red cells with the redox dye brilliant cresyl blue.

In the propositus, hemoglobin Pyrgos coexists with hemoglobin S. Whether

Table 2. Amino Acid Composition of the βTp 10 (83–95) From Hb Pyrgos

Amino Acid Residue	β Pyrgos Tp 10 (83–95) Molar Ratios Found	β^A Tp 10 (83–95) Expected Ratios
Lysine	0.77	1
Histidine	0.73	1
Aspartic acid	1.93	1
Threonine	1.67	2
Serine	1.03	1
Glutamic acid	1.35	1
Glycine	—	1
Alanine	1.00	1
Cysteine*	—	1
Leucine	2.04	2
Phenylalanine	1.03	1

*Not found in the amino acid analysis. It was found to be present by special stain for sulfur on the peptide maps.

or not there is an interaction between these two hemoglobins is not known at the present time because in vitro studies, such as minimum gelling concentration and blood viscosity measurements, have not been done. However, clinically there is no evidence of interaction because the propositus, as well as the other two carriers are healthy and asymptomatic.

There are two other variants described to date with a substitution at the EF7 position. One is hemoglobin Stanleyville II with a substitution of a lysine for asparagine at the 78th position of the α chain.[10] This molecule seems to be stable, and there are no clinical effects attributed to this variant, even when it is inherited together with hemoglobin S.[11,12] This variant, like hemoglobin Pyrgos, does not seem to have any interaction with hemoglobin S at the clinical level. The other, hemoglobin Ta-Li, has a cysteine in place of a glycine at the 83rd position of the β chain.[13] This mutation, too, does not seem to cause any instability in the molecular structure, and the carriers are hematologically and clinically normal.

The carriers of hemoglobin Pyrgos demonstrate mild red cell abnormalities on the peripheral blood smear. Whether these changes are due to the presence of this hemoglobin or the inheritance of another gene, such as α-thalassemia, is difficult to state with certainty at present. Iron deficiency can be excluded as the cause of these changes because the serum iron of all carriers was within normal limits and because the erythrocyte abnormalities remained after a trial of oral iron therapy.

The amount of hemoglobin Pyrgos in the hemolysates of the two heterozygotes for hemoglobin A and Pyrgos was greater than hemoglobin A, as determined by the chromatographic separation of the two hemoglobins. This finding was thought to be due to the elution of the hemoglobin A_{Ic} chromatographic fraction together with hemoglobin Pyrgos from the CM-cellulose column. However, the difference in the amount of the two hemoglobins seems to be real, because there is a larger amount of β Pyrgos chain eluted from the CM-cellulose-urea column during the chain separation of the globins of the unfractionated hemolysate from these carriers than β^A.

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