Hemoglobin L Ferrara in a Jewish Family Associated with a Hemolytic State in the Propositus

By Ronald L. Nagel, Helen M. Ranney, Thomas B. Bradley, Alan Jacobs and Linda Udem

The association of congenital nonspherocytic hemolytic anemia with the presence of certain unstable hemoglobins has been recognized in numerous patients. The instability of these hemoglobins has been manifested by precipitation on heating in vitro and by the formation of Heinz bodies with premature destruction of erythrocytes in vivo. The molecular basis for the instability of certain of these hemoglobins has recently been reviewed by Perutz and Lehmann.

The present report is concerned with the association of a longstanding hemolytic state in only one of seven members of a Jewish family heterozygous for a genetically determined hemoglobin variant, hemoglobin L Ferrara (Hb LF) in which glycine replaced aspartic acid at residue α47, interhelical residue αCD5. Hemoglobin L Ferrara has been described previously (in an Ashkenazi Jew) as hemoglobin Beilinson; one of three family members with Hb Beilinson apparently had leukemia, and the other two were not anemic.

The present report includes 1) studies of a Jewish family with Hb L Ferrara, 2) studies of some properties of Hb L Ferrara and 3) the results of attempts to relate the presence of the abnormal hemoglobin to the hemolytic state observed in the propositus.

Materials and Methods

1) Characterization of Hemoglobin L Ferrara

The abnormal hemoglobin was characterized by the methods of Clegg, Naughton and Weatherall with modifications described previously.

2) Other Studies

Phase contrast microscopy of erythrocytes suspended in saline, and of lysed erythrocytes was utilized for study of Heinz bodies. Oxygen equilibria were performed at 10 C. by

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the spectrophotometric technic of Allen, Guthe and Wyman\textsuperscript{a} with minor modifications. Reactive sulphydryl groups were titrated with p-hydroxymercuribenzoate according to the method of Boyer\textsuperscript{b} as modified by Benesch and Benesch.\textsuperscript{11} Tyrosine titration was performed as described previously.\textsuperscript{12} Heat stability tests were performed at 50 C. and 55 C. Hemoglobin A and Hb LF from several members of the pedigree were separated by starch block electrophoresis with veronal buffer 0.05 M pH 8.6 and eluted with the same buffer. An appropriate volume of Hb solution at a concentration of about 3 \( \times \) \( 10^{-5} \)M was placed in a temperature regulated water bath. Aliquots were removed at several intervals and immediately diluted to \( \frac{3}{4} \) of the original concentration with cold veronal buffer (pH 8.6). The hemoglobin concentration of the supernatant was determined after centrifugation and conversion to cyanmethemoglobin.

**RESULTS**

**A. Clinical and Hematologic Findings**

1) **Family studies** (Fig. 1) Propositus (II-5) - J.J., a 41 year old male of Jewish ancestry, was referred to the Heredity Clinic in 1964, for evaluation of an electrophoretically abnormal hemoglobin which had been found at the Columbia-Presbyterian Medical Center. The patient had had jaundice and mild hepatosplenomegaly at the age of 17; at that time, hemoglobin was 13.0 Gm. per cent; reticulocytes 9.4 per cent; serum bilirubin 10.7 mg. per cent (indirect); alkaline phosphatase 3.8 BU; osmotic fragility increased: beginning hemolysis at 0.475 with normal control beginning at 0.425. From age 17 to 23 years, he had increasing splenomegaly, a persistently high reticulocyte count and mild jaundice. A splenectomy was performed in 1946 without notable change in his hematologic status, and in 1950, he underwent a cholecystectomy.
During annual clinic visits in the ensuing 14 years, low grade icterus, nearly normal hemoglobin values, reticulocytosis of about 10 per cent and persistent leucocytosis were found. He was generally free of symptoms. The laboratory tests on his yearly evaluation in 1964 showed an abnormal hemoglobin migrating in the position of Hb S. Sickling was not observed. A moderate decrease in erythrocyte survival was demonstrated with $^{51}$Cr labeling ($T % = 23$ days; normal $T % = 30$ days). The father of the propositus (I-2) was asymptomatic, hematologic studies as well as $^{51}$Cr erythrocyte life span were normal. The only data available on the mother of the propositus (I-1) had been obtained in 1959: hemoglobin was 10.6 Gm. per cent, slightly increased erythrocyte fargility and a reticulocyte count of 3.4 per cent were noted. She was dead when the present studies were undertaken. All other heterozygotes of the pedigree were asymptomatic.

2) Other Hematologic Data. Doctor William N. Valentine and Dr. Ernst Jaffé carried out enzymatic assay on red cells of the propositus. Values for glucose-6-phosphate dehydrogenase, phosphoglucuronate dehydrogenase, glutathione reductase, hexokinase, glucosephosphate isomerase, phosphofructokinase, fructosephosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase, phosphoglycerate kinase, monophosphoglyceromutase, phosphopyruvate hydratase (enolase), pyruvate kinase, lactate dehydrogenase, GSH peroxidase, glyoxalase, acetylcholinesterase and pentose shunt enzymes (PETT) were either normal or increased as would be expected from the consistent reticulocytosis. Reduced glutathione, GSH stability test, and the assay for monophosphoglyceromutase plus 2-3 diphosphoglycerate were also normal.

The erythrocyte lipid profile studied by Dr. Eugene L. Gottfried on red cells of the propositus demonstrated that the total lipid, lipid phosphorous and cholesterol were symmetrically increased, consistent with an increased percentage of large young cells. The distribution of individual phospholipids (phosphatidyl choline, ethanolamine phosphatide, phosphatidyl serine), determined by quantitative thin-layer chromatography, showed only small deviations from the average normal values. Similar patterns have been seen in other patients with significant reticulocytosis.

Intact and lysed red cells were examined for Heinz bodies with phase microscopy. Fresh red cells of the propositus contained only rare Heinz bodies, but in red cells stored for three days at 4 C., abundant Heinz bodies were observed. In the same tests performed on the erythrocytes of the sons of the propositus (III-5 and III-6 of Fig. 1) no inclusion bodies were demonstrable.

Seven members of the family were heterozygous for Hb LF. The major abnormal component in the hemolysates of these individuals was indistinguishable from Hb S on starch gel electrophoresis at pH 8.6 in Tris-Borate buffer (Fig. 2). In these hemolysates Hb $\mathcal{A}_2$ was decreased and the presence of a second minor component more basic than normal Hb $\mathcal{A}_2$ (putatively $\alpha^{LF}_2 \delta_2^0$) suggested that the abnormal major component was an $\alpha$ chain variant. The major abnormal component amounted to about 20 per cent of
Fig. 2.—Vertical starch gel electrophoresis, Tris-borate buffer, pH 8.6. Benzidine stain. Unfractionated hemoglobin samples from two individuals, one heterozygous for hemoglobin S, the other for hemoglobin L Ferrara were applied to the gel in 1 per cent and 10 per cent concentrations. Note that the variant of hemoglobin $\alpha^L F \delta^A 2^A$, the most basic component, is readily visualized only at the higher concentrations. At both hemoglobin concentrations, the proportion of normal hemoglobin $A_2 (\alpha^A \delta^A \lambda^A)$ appears to be significantly decreased in the hemolysate of the hemoglobin LF heterozygote when compared with the hemolysate of the hemoglobin S heterozygote.

the total hemoglobin in all heterozygotes. The average proportion of Hb A$_2$ in heterozygotes for Hb L Ferrara who had no evidence of hemolysis was 1.6 per cent; the more basic minor component ($\alpha^L F \delta^A 2^A$) constituted less than 0.5 per cent of the hemoglobin.

B. Chemical and Functional Findings on Hb L Ferrara

1) Structural characterization of Hb L Ferrara. A peptide map of alpha LF chains is shown in Figure 3. The normal peptide $\alpha A$ T-6 was missing but was replaced by a peptide ($\alpha LF$ T-6) which migrated further toward the cathode. Amino acid analyses of the acid digests of the variant peptide and of $\alpha A$ T-6 demonstrated the absence of aspartic acid and the presence of two (instead of the normal one) glycine residues in the variant peptide. Thus this abnormal hemoglobin (originally designated Hb Columbia) is identical with hemoglobin L Ferrara, studied by Baglioni, in which glycine is sub-
Fig. 3.—Peptide maps of α chains from hemoglobin LF. Small circle indicates site of application of tryptic digest. High voltage electrophoresis at 3000 V for 105 minutes in 1.25 per cent pyridine, 1.25 per cent acetic acid pH 4.7. Ascending chromatography in BAWP (see text). Arrow indicates peptide αT6 which is displaced toward the cathode in Hb LF (Kindred 161). Dotted circle indicates the site of normal αT6 which was absent in maps of αL.

stituted for aspartic acid at α47 (CD5). No specific role is assigned to CD5 and glycine occupies this position in normal β chains.

2) Tyrosine titration. The spectrophotometric titration of tyrosyl hydroxyl groups of Hb LF and Hb A at 245 mμ is depicted in Figure 4. An apparent pK of 10.6 was found for eight tyrosyls: the remaining four tyrosyls have an “abnormal” pK; they are probably buried in the molecule or participating in hydrogen bonds. No differences in the environment of the tyrosyls of Hb A and Hb LF were demonstrated by this method.12

3) Titration of -SH groups. Only two of the six cysteine residues of the Hb LF tetramer were titratable with p-hyrdoxymercuribenzoate. A similar result was reported for Hb A by Benesch and Benesch.15

4) Heat stability. Hemoglobin LF and Hb A did not differ significantly in the heat stability test at 50 C. described by Dacie and co-workers.1 Since Charache and Mondzac16 found Hb Hasharon (Hb Sinaiα2 β2) to be more unstable than Hb A at 60 C., the effect of higher temperatures on Hb LF was studied. Heat instability of Hb LF was observed at 55 C. (or higher). The results of heat stability testing at 55 C. are shown in Figure 5. After one hour, 60-65 per cent of the Hb LF added was still present in the supernate, compared with 90 per cent in the case of Hb A.

5) Oxygen equilibria of Hb L Ferrara. The oxygen equilibria of dilute solutions of Hb LF and Hb A in 0.1 M phosphate buffers at 10 C. did not differ significantly with respect to oxygen affinity, cooperative interactions (n from Hill’s equation), or Bohr effect. At pH 7.38, value of p ½ for Hb LF was 1.84 mm. Hg with n value of 2.7; at pH 7.41, corresponding values for Hb A were 2.32 mm. Hg and 2.7.

**DISCUSSION**

The relationship of Hb L Ferrara to the accelerated erythrocyte destruc-
Fig. 4.—Spectrophotometric titration of tyrosyl residues of hemoglobin A and hemoglobin L Ferrara. Temperature: 25°C. Ionic strength: 0.1. ● = Hb L Ferrara; ○ = Hb A; Δε = Graphic extrapolation to 0 time of time dependent observations. The curve is biphasic, and its first part describes a typical titration curve which can be accounted for by the following equation:

\[ \text{pH} = \text{pK} + \log \frac{\Delta \varepsilon}{\Delta \varepsilon_{\text{max}} - \Delta \varepsilon} \]

The curve shown in Figure 4 can be described by this equation when \( \Delta \varepsilon_{\text{max}} \) is 92,000 and pK is 10.60.\(^1\) The second part of the curve corresponds to the rapid titration of tyrosyl groups above pH 11.50, probably due to the uncovering of tyrosyl side chains otherwise unavailable to the solvent. In this portion of the titration curve the absorption readings become time dependent in Hb LF as in Hb A.\(^1,2\) Above pH 12 the absorption readings again become time independent. Dividing \( \Delta \varepsilon_{\text{max}} \) by the \( \Delta \varepsilon \) of one tyrosyl (12,100) showed that eight tyrosyls per molecule of HB (MW: 64,500) titrated with an apparent pK of 10.60. There remained four tyrosyl residues, of the 12 known by amino acid analysis, with "abnormal" pK.

The clinical significance of greater instability of Hb LF at 55°C is uncertain since these data are not available for most Hb variants. Furthermore, evidence of hemolysis was lacking in six family members heterozygous for Hb LF. A similar variability in clinical findings has been encountered in the other recognized \( \alpha \) variant, Hb Hasharon (which has also been found in Jewish families)\(^16-18\); no clinical abnormalities were noted by Schneider and co-workers,\(^18\) whereas hemolysis was obvious in one of the patients of Charache and Mondzac.\(^16\) The latter authors\(^16\) from studies of isotopically labelled hemoglobin from carriers of Hb Hasharon...
Fig. 5.—The heat stability of hemoglobin L Ferrara and hemoglobin A at 55 C., pH 8.6, 0.05M veronal buffer. The hemoglobin remaining in solution is plotted against time. —— = Hb A; —— = Hb LF (II-5); o o = Hb LF (III-5); ○○ = Hb LF (III-6).

concluded that the abnormal hemoglobin was denatured and removed from aging red cells.

In our studies of a family with Hb LF, the presence of Heinz bodies in incubated red cells of the propositus but not of asymptomatic carriers suggested the possibility of an additional erythrocyte defect in the propositus. No additional abnormality was revealed by studies of red cell enzymes or lipids however. The variant hemoglobin of the present study, \( \alpha^{47} \), as well as of the patient of Charache and Mondzac, \( \alpha^{47} \), was found during the evaluation of patients with hematologic disorders. It is possible that the presence of the hemoglobin variant is an incidental finding, unrelated to accelerated erythrocyte destruction of undefined etiology. Alternatively there remains the possibility that a substitution at \( \alpha^{47} \) renders the hemoglobin unstable in the presence of an otherwise "silent" red cell factor or defect.

Summary

A Jewish family in which Hb L Ferrara (\( \alpha^{47} \)) occurred is reported. Studies of some of the properties of this hemoglobin demonstrated that its oxygen equilibria, number of readily reactive -SH groups, and spectrophotometric tyrosine titration were indistinguishable from Hb A. Nevertheless, Hb LF was more unstable than Hb A at 55 C. The propositus had accelerated blood destruction although six other heterozygotes for Hb LF did not. A second defect in red cell enzymes or red cell lipids of the propositus was not demonstrable with the technics used but the possibility that the simultaneous occurrence of Hb LF and an otherwise "silent" red cell defect may lead to a hemolytic state remains an attractive explanation. The data provided by this family study did not permit a definite conclusion about the relationship of
clinically evident hemolysis in the propositus to the presence of the abnormal hemoglobin.

SUMMARIO IN INTERLINGUA

Es reportate un familia judee in le qual occurreva Hb L Ferrara (α^2, β^2). Studios de certes del proprietates de iste hemoglobina demonstrava que su equilibrios de oxygeno, su numero de prestemente reactive gruppos SH, e su titration spectrophotometric de tyrosina esseva indistinguibile ab le correspondente characteristicas de Hb A. Nonobstante, Hb LF esseva plus instabile que Hb A a 55 C. Le proposito habeva un accelerate destruc-

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