Hemoglobin D Los Angeles in Two Caucasian Families: Hemoglobin SD Disease and Hemoglobin D Thalassemia

By Rose G. Schneider, Satoshi Ueda, Jack B. Alperin, William C. Levin, Richard T. Jones, and Bernadine Brimhall

Hemoglobin D Los Angeles, \( \alpha_2\beta_2^{121 \text{id}} \) (Or D Punjab) is one of the more common hemoglobin variants, since it occurs in about 3 percent of the Sikhs of the Punjab and sporadically in other ethnic groups, particularly those that have had considerable contact with India. In electrophoresis and chromatography, this variant is indistinguishable from hemoglobin S; however, it lacks the insolubility of hemoglobin S in the reduced form and, therefore, the ability to cause sickling. It also differs from hemoglobin S on electrophoresis in citrate agar, in which medium it moves like hemoglobin A. Several structurally different hemoglobin variants, such as D Ibadan, \( \alpha_2\beta_2^{121 \text{id}} \) or \( \alpha_2\beta_2^{121 \text{ly}} \), share these properties, while still others, such as L, P, and several varieties of G, are similar enough to be easily mistaken for hemoglobin D or for each other. Thus, several hemoglobins originally called D were later found identical with G Philadelphia, which, in turn, was first described as Stanleyville I.5

Although a number of reports of sickle cell hemoglobin D disease have appeared, there are only a few in which the hemoglobin D has been precisely identified. Sturgeon et al.6 described a family of English, Irish, and American Indian ancestry carrying the genes for hemoglobins S and D Los Angeles.1 Two siblings in this family had inherited a gene for each of these variants and both suffered from a moderate hemolytic anemia. A similar case was described by Smith and Conley,7 and the hemoglobin D Los Angeles was identified by Baglioni.8 In contrast, the simultaneous presence of hemoglobins S and D Ibadan9 in two members of a Nigerian family, was not associated with any disease.
HEMOGLOBIN D LOS ANGELES

Hemoglobin D thalassemia disease has been reported several times, but the structure of the hemoglobin D in these cases was not determined. The present report deals with hemoglobin D Los Angeles found in two Caucasian families of widely different ethnic origins, in one family together with hemoglobin S and in the other with β-thalassemia.

METHODS

Hemograms were obtained with standard techniques. Erythrocytes and leukocytes were enumerated in an electronic counter. Bone marrow was stained with Kingsley’s stain and Prussian blue. The methods of Peters et al. were used to measure serum iron and total iron-binding capacity. Erythrocyte survival time was measured with 51Cr-labeled autologous red cells, and ferrokinetic measurements were performed with autologous plasma labeled with 59Fe. Hemolysates were prepared in the usual manner and examined electrophoretically on starch gel and cellulose acetate, tris ethylene diamine tetraacetic acid borate (TEB) buffer, pH 8.6, and on citrate agar, pH 6.2. Fetal hemoglobin values were determined by the alkali denaturation method of Betke et al. and quantitative estimations of other hemoglobins were obtained by chromatography on diethyl amino ethyl cellulose (DEAE).

Purified hemoglobin components were converted to globin by treatment with cold acid-acetone, and the polypeptide chains were separated by chromatography on carboxymethyl cellulose in 8 M urea. The cysteinyl residues of the β chain were aminoethylated with ethylenimine as described elsewhere. The purified β chain of hemoglobin D was hydrolyzed with trypsin (Worthington 2X crystallized), and the tryptic peptides were separated by automatic chromatography on columns of Spinco 15A resin.

The abnormal peptide was rechromatographed on a column of Aminex 50W-X2 resin (Bio-Rad Inc.), with a linear gradient of pyridineacetate buffers, pH 3.1 and 0.2M to pH 3.6 and 0.67M. The effluent from this column was subjected to automatic alkaline hydrolysis followed by neutralization and reaction with ninhydrin.

Quantitative amino acid analyses were made after hydrolysis with 6N HCl in vacuo at 110 C. for 22 hr., using a Spinco amino acid analyzer modified to give a tenfold increase in sensitivity. A special enzymatic hydrolysis of approximately 0.1 μmoles of the abnormal peptide was effected with 25 units of Aminopeptidase M (Henley and Co., New York) in 0.5 ml. of tris buffer at pH 8 for 16 hr. at 37 C.

CASE REPORTS

Sickle Cell Hemoglobin D Disease

G. de L., a 19 year old Caucasian male, was first examined at the University of Texas Medical Branch in 1963. He had experienced repeated episodes of pain of variable intensity in his back, chest, abdomen, and extremities since childhood, and jaundice and anemia were observed when he was 13 years old. Neither the liver nor spleen were palpable. His hemoglobin concentration was 7 to 8 Gm./100 ml. Target cells and sickle cells were prominent on blood smear examination. Total serum bilirubin measured 3.9 mg./100 ml., of which 1.3 mg. was direct-reacting bilirubin. He was readmitted in 1967 because of severe, generalized pain of four days duration. He stated that he suffered attacks of pain at four to six month intervals, but he denied dyspnea and exercise intolerance. Except during painful crises, he had worked regularly. He was in obvious distress, but the vital signs were normal. The spleen was not palpable, but the liver extended 5 cm. below the costal margin. There were no ulcerations nor healed scars on the lower extremities. Fluids and analgesics were administered, and by the third hospital day the pain had abated. Pneumonia developed during the course of this hospitalization, but cleared promptly in response to antibiotics. The hematologic data are presented in Table 1. and the blood smear is shown on Figure 1.
Table 1.—Hematologic Data on the Two Patients With Hemoglobin D Los Angeles

<table>
<thead>
<tr>
<th></th>
<th>G. DeL. (Sickle Cell Hb D Disease)</th>
<th>H. W. (Hb D Thalassemia Disease)</th>
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</thead>
<tbody>
<tr>
<td>Hemoglobin (Gm./100ml.)</td>
<td>9.4</td>
<td>9.1</td>
</tr>
<tr>
<td>Erythrocytes (10⁶/mm.)</td>
<td>3.6</td>
<td>4.6</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>29</td>
<td>28</td>
</tr>
<tr>
<td>Mean cell volume (µ³)</td>
<td>81</td>
<td>60</td>
</tr>
<tr>
<td>Mean cell hemoglobin (pg)</td>
<td>26</td>
<td>20</td>
</tr>
<tr>
<td>Mean cell hemoglobin concentration (%)</td>
<td>33</td>
<td>33</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>14.4</td>
<td>4.1</td>
</tr>
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- Hypochromia
- Target cells
- Basophilic-stippling
- Microcytes
- Macrocytes
- Sickle cells
- Positive
- Decreased

Analysis of the Hemolysates: In zone electrophoresis in alkaline buffers and in chromatography, the patient’s hemolysate is indistinguishable from that of sickle cell anemia, but in citrate agar electrophoresis it resembles that of sickle cell trait. The patient’s mother, who is of Spanish origin, has sickling erythrocytes and an AS hemoglobin pattern. His father, who is Mexican, has a negative sickling test, but on electrophoresis in alkaline buffers and on chromatography his hemolysate resolves into two approximately equal fractions resembling hemoglobin A and S. In citrate agar electrophoresis, however, there is only one major component, moving like hemoglobin A. No hemoglobin abnormality was found in the one examined sibling. The electrophoretic patterns are shown in Figures 2a and 3a, and the quantitative analyses of the hemoglobin are presented in Table 1.

Hemoglobin D Thalassemia

H.W., a 57 year old woman of English, Irish, and Scotch ancestry, was admitted to the hospital for evaluation of anemia. She denied weakness, easy fatigability, exercise intolerance, and dyspnea. At age 12 years she had rheumatic fever, and at age 37 she had an uncomplicated pregnancy. Thirteen years before admission her spleen was observed to extend 5 cm. below the costal margin. At that time, her hemoglobin measured 12.4 Gm./100 ml., and the blood smear examination revealed hypochromic erythrocytes and numerous target
Hb Types

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<tr>
<td>AS</td>
<td>FA</td>
</tr>
<tr>
<td>AD (Father)</td>
<td>DA (H. W.)</td>
</tr>
<tr>
<td>SS</td>
<td>SS</td>
</tr>
<tr>
<td>SD (G. DeL.)</td>
<td>AD (Son)</td>
</tr>
</tbody>
</table>

Fig. 2—Cellulose acetate electrophoresis, TEB buffer, pH 8.5, of hemolysates of (a) G. DeL. (sickle cell hemoglobin D disease) and (b) H. W. (hemoglobin D thalassemia disease).

A reticulocyte count was not reported. Four months prior to admission, she consulted her family physician because of an episode of gastroenteritis. Splenomegaly was again noted, and her hemoglobin level was 5.4 Gm./100 ml., but no other laboratory tests were performed.

On admission, the patient's vital signs were normal, and icterus was not apparent. The spleen extended 10 cm. below the left costal margin. Consulting cardiologists thought the patient had aortic stenosis and regurgitation, and a phonocardiogram was consistent with this opinion. The fecal excretion of urobilinogen, based upon a 96 hr. stool collection, was 341 mg./day. Hepatic and renal function appeared normal, as did skeletal roentgenograms.
cholecystogram revealed radiopaque gall stones. In the sternal marrow, there was an intense normoblastic erythroid hyperplasia, and on staining with Prussian blue many particles of hemosiderin were demonstrable. The hematologic data are shown in Table 1, and Figure 4 shows a typical blood smear.

Since leaving the hospital, the patient takes care of her home and participates in a family business. Hemoglobin concentrations are 8.5 to 9.5 Gm./100 ml. The oral administration of ferrous sulfate, 300 mg. three times a day for one month, failed to produce hematologic improvement, but transfusions have not been necessary.

Analysis of Hemolysates: In chromatography and on zone electrophoresis in alkaline buffers (Fig. 2b), the patient's hemolysate resolves into about 80 per cent hemoglobin D, the remainder being distributed almost equally between hemoglobins A, F, and A2 (Table 1). However, in citrate agar electrophoresis the hemoglobin moves like hemoglobin A (Fig. 3b).

The patient's parents and all of their siblings are dead. Her son and a paternal first cousin have hemoglobin D trait. No hemoglobin abnormalities were found in her brother and five
maternal first cousins. All of the examined family members have normal hemograms, normal erythrocyte morphology, and normal quantities of hemoglobins A2 and F.

**Chemical Characterization of Hemoglobins**

The slow-moving hemoglobin from one member of each described family was treated to obtain the aminoethylated beta-chain fraction. This was hydrolyzed with trypsin and applied to a column of Spinco 15A resin, yielding a chromatogram which did not appear to differ from that of the normal beta-chain of hemoglobin A. However, when the zone which normally contains peptides beta-13 and beta-3 (see Zone II, Fig. 4, Ref. 22) was rechromatographed on Dowex 50W-X2 resin with alkaline hydrolysis of the eluate, there was no beta-13 peak in the expected position, but rather a new peak that emerged much earlier (Zone I, Fig. 5). The peptide in the abnormal Zone I, after hydrolysis with 6N HCl, gave an amino acid analysis identical to that of the normal beta-13 peptide (Table 2). The normal peptide consists of amino acid residues 121 through 132 of the beta chain in the sequence: GluPheThrProProValGlnAlaAlaTyrGlnLys.

The amino acid residues liberated by the action of aminopeptidase M on the two peptides are listed in Table 2. The enzyme cleaved Glu and Phe from the N-terminal end of normal beta-13, but did not release either of these from the abnormal beta-13. In neither case was free proline found in the digest; however, amino acids at the carboxy-terminal portion of the peptides were released, with lysine and tyrosine being completely removed. This hydrolytic action for aminopeptidase is not typical but has been observed with several other peptides.

**Discussion**

Both examples of the variant described here exhibit the unique chemical behavior of hemoglobin D Los Angeles, alpha2beta2^{121}{Glu}. The substitution of a glutaminyl residue for the more acidic glutamyl residue gives hemoglobin D Los Angeles an electrophoretic mobility (in alkaline buffers) slower than that
of hemoglobin A. In peptide chromatography in acid medium, the abnormal peptide is eluted more rapidly than the corresponding peptide of hemoglobin A. This peculiarity of the abnormal peptide is due to the loss of the free NH₂ terminal group which, under the conditions of the chromatography, undergoes a cyclization with the y amide group of the glutaminyl residue to produce a residue of pyrrolidone carboxylic acid. On hydrolysis with HCl, this is converted to glutamic acid, so that the amino acid composition of the abnormal peptide now appears identical with that of the corresponding normal one. However, after hydrolysis with aminopeptidase M, the normal and abnormal peptides yield different amino acid analyses, since the pyrrolidone carboxylic residue will not serve as substrate for enzymatic hydrolysis.

No previous reports of the occurrence of hemoglobin D have appeared from Mexico, and sickle cell trait is not common in Spain. Nevertheless, the patient described here has inherited a gene for hemoglobin D Los Angeles from his Mexican father and one for hemoglobin S from his Spanish mother. His most prominent symptom is severe pain in the extremities, back, and abdomen. Of the three previously reported patients with sickle cell hemoglobin D Los Angeles disease, two frequently experienced severe painful crises, but the third had infrequent bouts of pain as a child and was clinically well as an adult. All three patients, as well as the one described here, suffered from hemolytic anemia, with hemoglobin concentrations of 9.1 to 9.9 Gm./100 ml. In all four, the erythrocyte morphology is distinctly abnormal, with numerous sickle cells, target cells, and polychromatophilia. Indeed, in the absence of genetic data or special studies of the hemoglobin, these patients might be thought to have either sickle cell anemia or sickle cell β-thalassemia disease.

In contrast, the heterozygotes for both hemoglobins S and D Ibadan were asymptomatic, and their erythrocyte morphology appeared normal. Only about 20 per cent of their hemoglobin was of the sickling type, whereas the heterozygotes for both hemoglobins S and D Los Angeles have approximately equal

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**Table 2.—Amino Acid Residues Obtained After Hydrolysis of βT-13 Peptides**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Found with 6N HCl</th>
<th>Expected with Aminopeptidase M</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Abn. βT-13</td>
<td>Abn. βT-13</td>
</tr>
<tr>
<td></td>
<td>Nor. βT-13</td>
<td>Nor. βT-13</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.01</td>
<td>1.07</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.97</td>
<td>*</td>
</tr>
<tr>
<td>Glutamine</td>
<td>1.02</td>
<td>1.53</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>3.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Proline</td>
<td>2.07</td>
<td>1.34</td>
</tr>
<tr>
<td>Alanine</td>
<td>2.01</td>
<td>1.00</td>
</tr>
<tr>
<td>Valine</td>
<td>1.03</td>
<td>.37</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.96</td>
<td>1.00</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.94</td>
<td>.92</td>
</tr>
</tbody>
</table>

*Threonine and glutamine are eluted in the same place under the conditions used for amino acid analysis.*
quantities of the two hemoglobin variants. At least nine other patients with sickle cell hemoglobin D disease have been described, some of whom have the clinical and hematologic features similar to those of our patient; but since the hemoglobin D was not identified, it is not certain that these involve hemoglobin D Los Angeles.

Hemoglobinopathies are infrequently seen in Anglo-Saxon populations, but sporadic instances both of $\beta$ thalassemia and of hemoglobin D trait have been found in Great Britain. The patient with hemoglobin D Los Angeles $\beta$-thalassemia described here is of English, Irish, and Scotch ancestry. She exhibits splenomegaly and moderate hemolytic anemia, with erythrocyte morphology typical of thalassemia. Her hemoglobin pattern is characteristic of heterozygosity for both $\beta$-thalassemia and a $\beta$-chain-hemoglobin variant. Other cases of hemoglobin D thalassemia have been described in various ethnic groups such as Persian, Indian, and Sikh. These patients suffered from varying degrees of hemolytic anemia, but, again, the hemoglobin D was not precisely identified, so that variants other than hemoglobin D Los Angeles may have been involved.

The mechanism by which hemoglobin D Los Angeles exerts its pathologic effect is not known, since its solubility is not abnormal, nor does the $O_2$-dissociation curve of the blood of patients with hemoglobin D Los Angeles trait differ from the normal. However, a slight increase in $O_2$ affinity was found in the isolated variant when compared with isolated hemoglobin A. The most severe pathologic effect of hemoglobin D Los Angeles appears in its heterozygous combination with hemoglobin $S$, and erythrocytes containing both of these hemoglobins sickle almost as readily as do those of patients homozygous for hemoglobin $S$. The homozygous state for hemoglobin D Los Angeles presents a milder clinical course, which is very similar to that of our patient with hemoglobin D Los Angeles $\beta$-thalassemia.

The variation in clinical symptomatology associated with the presence of two of the known types of hemoglobin D (Los Angeles and Ibadan) indicates the need for determination of the specific amino acid substitution in the rarer hemoglobin variants, so that clinical and hematologic abnormalities may be correlated with specific molecular defects.

**Summary**

Data are presented on two Caucasian families with hemoglobin D Los Angeles, $\alpha_2\beta_2^{\text{D Los Angeles}}$. In one family, the mother, of Spanish origin, has sickle cell trait and the father, of Mexican origin, has hemoglobin D trait. One child has sickle cell hemoglobin D disease and suffers from a moderately severe hemolytic anemia. In the other family, of English, Scotch, and Irish ancestry, one member has the hemoglobin DAF pattern of hemoglobin D $\beta$-thalassemia disease and suffers from moderate hemolytic disease.

**SUMMARIO IN INTERLINGUA**

Es presentate dados relative a duo familias de raccia blanc con hemoglobina D Los Angeles $\alpha_2\beta_2^{\text{D Los Angeles}}$. In un del duo familias, le matre (de origine espaniol) ha le character de cellulas falciforme, e le patre (de origine mexican) ha le character de hemoglobina D.
Un del prole ha morbo de hemoglobina D a cellulas falciforme e suffre de un moderate-mente sever anemia hemolytic. In le altere familia (de origine anglese, scotic, e irlandese) tin membro ha le configuration hemoglobinic DAF de morbo -thalassemic a hemoglobina D e suffre de un moderate morbo hemolytic.

ACKNOWLEDGMENT

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