A New Abnormal Hemoglobin O Padova, α 30 (B11) Glu → Lys, and a Dyserythropoietic Anemia With Erythroblastic Multinuclearity Coexisting in the Same Patient

By L. Vettore, G. De Sandre, E. E. Di Iorio, K. H. Winterhalter, A. Lang and H. Lehmann

A patient with a not previously described abnormal hemoglobin (α30Glu−→Lys) and dyserythropoietic anemia with erythroblastic multinuclearity is reported. The heat stability and the functional properties of the new abnormal hemoglobin, named hemoglobin O Padova, are normal, although the replacement lies in the αβ interchain contact. The hemolytic condition, which was much improved by splenectomy, therefore appears to be linked to the hereditary erythroblastic multinuclearity similar to hereditary erythroblastic multinuclearity with positive acidified serum test (HEMPAS). In addition to the leading features observed in published cases of this entity, our case exhibited some immunologic peculiarities.

THE COEXISTENCE of two independent anomalies, both apparently genetically transmitted in the erythrocytes of the same subject, is very unusual.

The present report describes a woman of northern Italian origin, affected by a previously not observed abnormal hemoglobin, named Hb O Padova (α30Glu−→Lys) and by a dyserythropoietic anemia with erythroblastic multinuclearity similar to type II (according to Heimpel and Wendt).1 The structural physicochemical and functional properties of the abnormal protein are also reported.

MATERIALS AND METHODS

The hematologic investigations of the proposita were done by routine laboratory techniques.2 Special tests on the red cells were performed as indicated below.

For the demonstration of Heinz bodies, smears were obtained from fresh blood and after incubation with acetylphenylhydrazine.

Osmotic fragility tests were done according to the modifications by Dacie and Lewis2 of the original method by Parpart et al.3 on both fresh and preincubated blood (24 hr, 37°C).

Autohemolysis was checked after 48 hr incubation at 37°C, and the G6PD and PK levels were determined with standard methods.2 Acid lysis4 was tested with the serum of the propositus and four independent serum pools. The sera were depleted of complement by heating as needed.

The sugar-water test was carried out according to Hartmann et al.5 Agglutinability and lysis of the red cells of the patient were tested at 4°C and 37°C with anti-i, anti-I sera and also 50 sera of normal blood donors. The sera were used both before and after decomplementation by heat. The serum of the patient was tested against erythrocytes of 50 normal donors of group O and erythrocytes from ten different cord bloods (for technical details see Ref. 2). The sodium and potassium content of the erythrocyte was determined on fresh cells and after incubation at 37°C for 24 hr.4

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Bilirubin turnover was assessed according to Frezza et al.\textsuperscript{6} Fetal hemoglobin was measured as alkali-resistant hemoglobin.\textsuperscript{8}

**The Abnormal Hemoglobin**

The presence of an abnormal hemoglobin was demonstrated by electrophoresis on cellulose acetate strips\textsuperscript{2} and starch gel.\textsuperscript{9} Freshly prepared hemolysate was dialyzed against 0.05 \textit{M} Tris HCl, pH 8.3, containing 10\textsuperscript{-4} \textit{M} EDTA. The dialyzed hemolysate was applied to a DEAE-Sephadex column equilibrated with the above buffer. The column was then eluted by a linear 2-liter pH gradient going from pH 8.3 to 7.0. The fractions were checked for purity by starch-gel electrophoresis. Globin was prepared by precipitation in acid acetone.\textsuperscript{10} The globin was dissolved 1 mg/100 \\textmu l in 0.1 \textit{M} Veronal buffer, pH 8.0, containing 6 \textit{M} urea and 2\% v/v 2-mercaptoethanol. The electrophoresis was performed on cellulose acetate in a discontinuous Veronal TEB buffer, pH 8.0, containing 6 \textit{M} urea.\textsuperscript{11}

**Characterization of the Hemoglobin Variant**

Globin, prepared from the purified abnormal hemoglobin,\textsuperscript{11} was separated into its constituent \textalpha- and \textbeta-chains by ion-exchange chromatography on CM 23 cellulose.\textsuperscript{13} The \textalpha-chains were aminoethylated, extensively dialyzed against 0.5\% formic acid, and then lyophilized. Digestion of the aminoethylated \textalpha-chain with trypsin, fingerprinting of the soluble peptides at pH 6.4, and staining with ninhydrin and other reagents specific for arginine, histidine, methionine, tyrosine, and tryptophan were carried out as previously described.\textsuperscript{14} Where necessary, peptides were purified by electrophoresis at pH 3.5, 2.5 kV for 1 hr (pyridine, acetic acid, water, 1:10:89 v/v). The abnormal peptides were eluted from paper with 6N HCl, hydrolyzed for 24 hr at 108°C, and analyzed in a Locarte automatic amino acid analyser.

**Functional Properties of the Abnormal Hemoglobin**

Thermal stability was assessed by the method of Jacob and Winterhalter.\textsuperscript{15} Oxygen equilibria were obtained spectrophotometrically.\textsuperscript{16}

**RESULTS**

**Clinical and Hematologic Data of the Patient**

The propositus, born in 1940, was apparently healthy until she became icteric during her first pregnancy at 22 yr of age. During her second pregnancy at 27 yr of age the jaundice, which never had subsided completely, became more pronounced. At that time, a mild splenomegaly was observed for the first time. Also, the formerly rare painful crisis involving mainly the epigastric region became more frequent, and a moderate anemia was diagnosed. At her first admission to the hospital in 1969, anemia, icterus, and moderate splenomegaly were confirmed. The anemia (Hb 8.6 g) was slightly macrocytic, normochromic (MCV 104 \mu l, MCH 32 pg, MCHC 31\%). Marked anisocytosis and occasional target cells were observed. Furthermore, the peripheral smears revealed six orthochromatic erythroblasts per 100 nucleated elements. The bone marrow was extremely cellular, with an M/E ratio of 0.13. The erythroid maturation curve was shifted towards more mature elements (basophilic erythroblasts 4\%, polychromatophilic erythroblasts 14\%, orthochromatic erythroblasts 82\%). A few polychromatic erythroblasts and 16.4\% of the orthochromatic erythroblasts exhibited double or multiple nuclei (Fig. 1). Further hematologic data are reported in Table 1.

The survival time of erythrocytes was 24.7 days as calculated from the t \frac{1}{2}
ABNORMAL HEMOGLOBIN O PADOVA

Table 1. Time Course of Hematologic Data

<table>
<thead>
<tr>
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<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Ht (%)</td>
<td>28</td>
<td>30</td>
<td>25</td>
<td>40</td>
<td>37.5</td>
<td>34</td>
</tr>
<tr>
<td>Hb (g %)</td>
<td>9.6</td>
<td>8.64</td>
<td>10.24</td>
<td>11.40</td>
<td>9.00</td>
<td>14.20</td>
</tr>
<tr>
<td>RBC x 10^6/mmc</td>
<td>3.10</td>
<td>2.68</td>
<td>3.30</td>
<td>3.40</td>
<td>2.60</td>
<td>4.60</td>
</tr>
<tr>
<td>Reticulocytes (% RBC)</td>
<td>2.60</td>
<td>6.60</td>
<td>3.00</td>
<td>2.00</td>
<td>1.00</td>
<td>1.70</td>
</tr>
<tr>
<td>Total bilirubin (mg %)</td>
<td>2.10</td>
<td>2.20</td>
<td>2.46</td>
<td>3.26</td>
<td>3.15</td>
<td>1.50</td>
</tr>
<tr>
<td>Unconjugated bilirubin (mg %)</td>
<td>1.50</td>
<td>1.85</td>
<td>1.96</td>
<td>2.85</td>
<td>2.65</td>
<td>2.05</td>
</tr>
<tr>
<td>Serum iron (μg %)</td>
<td>134</td>
<td>200-280</td>
<td>245</td>
<td>110</td>
<td>155</td>
<td></td>
</tr>
<tr>
<td>TIBC (μg %)</td>
<td></td>
<td>326</td>
<td></td>
<td></td>
<td>326</td>
<td></td>
</tr>
<tr>
<td>RBC survival 51Cr (days)</td>
<td>24.7</td>
<td>45</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Bone marrow M/E ratio</td>
<td>0.13</td>
<td></td>
<td></td>
<td></td>
<td>0.81</td>
<td></td>
</tr>
<tr>
<td>Multinuclear erythroblasts (%)</td>
<td>16.4</td>
<td></td>
<td></td>
<td></td>
<td>19.3</td>
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</tr>
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</table>

determined by the 51Cr method, and the spleen/liver ratio gave a value of 2.09. The bilirubin turnover was 994 mg/24 hr (normal 234 ± 124.3/24 hr).

In view of the radiologically confirmed cholelithiasis, cholecystectomy was performed. Since the hemolytic anemia persisted, this was followed a year later by splenectomy, which resulted in a marked subjective and objective improvement. In particular, her reticulocyte count became near normal, her Hb value increased above 12 g, and the survival time of her erythrocytes was now 45 days (Table 1). Concomitantly, there was a decrease in the cellularity of her bone marrow (M/E ratio 0.81). In contrast, the maturation curve 22 mo after splenectomy was similar to presplenectomy values (basophilic erythroblasts 7%, polychromatophilic erythroblasts 17%, orthochromic erythroblasts 76%). Multinuclear orthochromic erythroblasts amounted to 19.3%.

Fig. 1. Bone marrow smear showing multinucleated erythroblasts.
Table 2. Acidified Serum Test on Propositus RBCs With Her Own Serum and Four Serum Pools, Each Derived From Three Different Healthy Donors

<table>
<thead>
<tr>
<th>Acidified Sera</th>
<th>Patient RBCs</th>
<th>Pool 1</th>
<th>Pool 2</th>
<th>Pool 3</th>
<th>Pool 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh Serum (F)</td>
<td>0</td>
<td>0</td>
<td>5.1</td>
<td>6.9</td>
<td>5.8</td>
</tr>
<tr>
<td>Heated Serum (H)</td>
<td>0</td>
<td>0</td>
<td>3.6</td>
<td>4.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>

F, fresh serum with complement.
H, heated serum without complement.

On freshly drawn cells no Heinz bodies were present before as well as after splenectomy. The cells, however, showed an increased sensitivity to phenylhydrazine treatment, exhibiting after that a great number of Heinz bodies. Neither the osmotic fragility nor the autohemolysis test revealed any abnormality. The levels of G6PD and PK were significantly increased, and intracellular electrolyte concentrations were normal. The results of acid lysis test are reported in Table 2, those of the serologic investigation in Table 3.

An abnormal hemoglobin, migrating like Hb A2 and amounting to 25% of the total hemoglobin, was detected in the hemolysate by cellulose acetate electrophoresis (Fig. 2). Starch-gel electrophoresis also revealed what appeared to be a slow-moving Hb A2, suggesting that the abnormal hemoglobin resulted from a mutation in the α-chain. This was confirmed by cellulose acetate electrophoresis of globin in 6M urea. The fingerprint of the soluble tryptic peptides from the amino ethylated α-chain (Fig. 3) showed that the mutation had occurred in residues α17–31, since α^TpIV was missing. The new peptide cathodal to α^TpIX had the same amino acid composition as α^TpIV (Table 4) except that one of the glutamic acid residues (α23, 27, or 30) had been replaced by a...

Table 3. Serologic Tests on Propositus RBCs* With Various Sera, and on Normal RBCs With Propositus Serum

<table>
<thead>
<tr>
<th>RBCs</th>
<th>Sera</th>
<th>Agglutination</th>
<th>Lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propositus</td>
<td>Anti-i normal sera</td>
<td>+ + +</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>(at 37°C or 4°C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>75% O group normal</td>
<td>Propositus</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>subjects (at 37°C)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30% O group normal</td>
<td>Propositus</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>subjects (at 4°C)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ten umbilical cord blood samples</td>
<td>Propositus</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*Blood group of propositus: O/CcDee/P1+.
Fig. 2. Cellulose acetate electrophoresis of the hemolysate showing Hb 0 Padova as a slow-moving band behind Hb A. Right: Hb A control. Left: Hb A + Hb 0 Padova. Discontinuous buffers: 0.12 M Tris-EDTA boric acid pH 9.1, 0.058 M barbiturate-barbituric acid pH 8.6; volts: 150; run: 2 hr; stain: Amido black.

residue of lysine. The neutral peptide, staining for histidine and tyrosine (Fig. 3), fixed the mutation at residue α30, since a peptide with this composition (Table 4, 17-30) could only have arisen from the α-chain if the glutamic acid at this position had been replaced by lysine. The presence of free arginine (Fig. 3) in the tryptic digest provided further confirmation that the abnormal hemoglobin was α30 (B11) Glu → Lys. The functional properties of the abnormal hemoglobin at 20°C are illustrated in Fig. 4. O2 affinity, cooperativity, and alkaline Bohr effect are perfectly normal. Between pH 6.8 and 8.9 the n value from the Hill equation remains at 2.9 ± 0.1. A minimal increase of the acid Bohr effect is also illustrated. No increased thermolability of unfractoned hemolysate or isolated Hb could be demonstrated.

Investigations on Relatives

For personal reasons numerous attempts to examine the father of the propositus or his children from a second marriage were unsuccessful. The mother and one of the two children (a boy) of the propositus both showed the abnormal hemoglobin in proportion similar to the patient. However, even the most careful investigation failed to reveal any signs of hemolytic anemia. Furthermore, the bone marrow of the mother was completely normal.

Fig. 3. Peptide chromatogram of the soluble tryptic peptides from the aminoethylated α-chain of Hb O Padova. Electrophoresis for 1 hr. 2.5 kV at pH 6.4 (pyridine: acetic acid: water, 25:1:224 v/v). Ascending chromatography for 18 hr. (isoamyl alcohol: pyridine: water, 6:6:7 v/v).
Table 4. Amino Acid Composition of the Peptides α1V (α17-31) and α17-30 Hb O Padova

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Hb O Padova</th>
<th>Hb A</th>
<th>Expected Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu</td>
<td>79.8</td>
<td>2.0</td>
<td>38.6</td>
</tr>
<tr>
<td>Gly</td>
<td>124.1</td>
<td>3.1</td>
<td>58.1</td>
</tr>
<tr>
<td>Ala</td>
<td>155.9</td>
<td>3.9</td>
<td>72.5</td>
</tr>
<tr>
<td>Val</td>
<td>40.3</td>
<td>1.0</td>
<td>18.5</td>
</tr>
<tr>
<td>Leu</td>
<td>41.8</td>
<td>1.0</td>
<td>20.8</td>
</tr>
<tr>
<td>Tyr</td>
<td>34.3</td>
<td>0.9</td>
<td>15.1</td>
</tr>
<tr>
<td>His</td>
<td>41.2</td>
<td>1.0</td>
<td>19.0</td>
</tr>
<tr>
<td>Lys</td>
<td>38.7</td>
<td>1.0</td>
<td>18.2</td>
</tr>
<tr>
<td>Arg</td>
<td>37.4</td>
<td>0.9</td>
<td>—</td>
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</table>

DISCUSSION

The uniqueness of the case presented here consists in the simultaneous occurrence of two relatively rare diseases. The abnormal hemoglobin exhibits an amino acid replacement not observed so far, αβGlu→Lys, thus conferring an electrophoretic mobility which at alkaline pH closely resembles the one of Hb O Indonesia. There also a Glu is replaced by a Lys, however, in position 116.17-19 In view of the electrophoretic similarity we would like to propose the name Hb O Padova for this new abnormal protein. Residue 30 of the α-chain (B11) lies in the αβ interchain contact, but apparently not in a critical region, since, surprisingly, the functional properties are virtually normal. In view of the fact that none of the stability tests were abnormal, Hb O Padova is not likely to be responsible for the hemolytic anemia. This idea is also born out by the lack of

![Fig. 4. Variation of log P0₂ 1/2 with pH for Hb O Padova. Conditions: 20°C, pH 6.0-7.5, 0.2 M phosphate buffer, pH 7.8-8.9, 0.2 M borate buffer. (---) Hb A, (-----) Hb O Padova.](image)
any signs of hemolysis in the mother and a child of the propositus who both
had the abnormal hemoglobin.

The second abnormality found in this patient—but not present in her two
relatives with Hb O Padova—appears to belong to the group described as
dyserythropoietic anemia with erythroblastic multinucularity and has several
features of type II according to the classification of Heimpel and Wendt with
positive acidified serum test (HEMPAS). This type of disorder has repeatedly
been described in the literature. In those cases the transmission of the ab-
normality appears to be recessive.

The present case has a number of features in common with the HEMPAS
cases of the literature:

The morphology of bone marrow and peripheral blood smears; the absence
of Heinz bodies in untreated blood and their abnormally copious appearance
after treatment with phenylhydrazine (the stability tests clearly show that this
is not due to an unusual instability of the abnormal hemoglobin);

the agglutination of the patient’s red cells with sera containing anti-i or anti-I;
the mild lysis of the patient red cells by acidified sera.

There are, however, also some differences:

over 90% of the sera of healthy donors agglutinated the red cells of our pa-
tient, in contrast to published reports where they react with only 30% of the
sera from healthy donors;

normal erythrocytes of the same blood group were lysed by serum of the
patient. At 37°C this phenomenon could be observed in 75% of all normal
erythrocytes tested, but at 4°C the lysis took place with only 30% of these same
erthrocytes;

the erythrocytes of our patient were lysed by acidified sera, even in the ab-
sence of complement, probably because of mechanical fragility due to cell
agglutination, since most normal sera strongly agglutinate the patient’s cells.

From the above observations we conclude that

1. As in other cases, the erythrocytes of our patient carry the antigens I and
i; moreover they have a further, different antigen reacting with a high pro-
portion of normal sera.

2. The serum of the patient has no anti-i, since no reaction is observed with
erthrocytes from cord blood. It contains an iso- or/and auto antibody reacting
independent of complement.

3. In contrast to most classic cases, together with the intramedullary he-
mosis, the peripheral lysis is of importance in our case: the greatly shortened
survival time of the erythrocytes was partially corrected by splenectomy; the
prolongation of the erythrocytic half-life markedly improved the patient’s
hematocrit, and the subicterus virtually disappeared. This was accompanied by
a decrease in cellularity of the bone marrow and an increase in the M/E ratio.
However, the multinuclearity and the serologic phenomena persisted after
splenectomy, as did the lysis from ineffective erythropoiesis, as testified by the
low reticulocyte count in presence of bone marrow erythroid hyperplasia.
ACKNOWLEDGMENT

The authors are indebted to Dr. G. Andolfatto Zaglia for performing the serologic tests.

REFERENCES

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