Detection of two Abnormal Hemoglobins, Hb Manitoba and Hb G-Coushatta, during Analysis for Glycohemoglobin (A1c) by High-Performance Liquid Chromatography

Shiu C. Wong, Maria Tesanovic, and Man-Chiu Poon

This report concerns the detection of two abnormal hemoglobins (Hb), Hb Manitoba and Hb G-Coushatta, during analysis for glycohemoglobin (Hb A1c). Blood samples from two diabetic patients, analyzed for Hb A1c by HPLC, were found to contain additional Hb peaks. The presence of an abnormal Hb was confirmed in both instances by hemoglobinopathy studies. Structural studies determined the two Hb variants to be Hb Manitoba (α2 102 Ser→Arg and Hb G-Coushatta (α2β2 22 Glu→Ala). The significance of the presence of an abnormal Hb in Hb A1c analysis is discussed.

Additional Keyphrases: chromatography, reversed-phase hemoglobin variants

Since the 1960s, it has been known that hemoglobin (Hb) fractions in erythrocytes are heterogeneous and contain various minor amounts of modified Hb (1). The minor glycohemoglobin form, Hb A1c, is a glycosylated form of Hb A and is found in increased amounts in diabetic patients (2, 3). Hb A1c has become very important in the monitoring of long-term control of diabetic patients, and numerous laboratory procedures, including micro-column chromatography, immunoassay, and electrophoresis, have been developed for its measurement (4–6). For quantification of several specimens, many clinical laboratories use either the commercially available affinity columns (7) or HPLC programs for analyzing Hb A1c (8). The latter procedure underestimates the quantity of Hb A1c in the presence of abnormal Hb because the minor fractions of these Hb variants, which can be glycated as well, will not be included in the total glycated fraction (9, 10). Here we describe two such variants.

Materials and Methods

Blood Samples

Blood samples for Hb A1c analysis were from two diabetic patients attending the Diabetic Day Care Clinic at the University of Calgary Health Sciences Centre. About 7 mL of blood was collected into Vacutainer Tubes (Becton Dickinson) containing EDTA as anticoagulant, and analyzed by the Clinical Chemistry Laboratory at the Foothills Hospital.

Blood for additional studies was obtained from the two diabetic patients who exhibited extra Hb peaks during Hb A1c analysis. Informed consent was obtained in both cases. Hemoglobin studies of these samples were performed by the Special Hematology Laboratory at the Foothills Hospital. Structural studies were performed by the Protein Chemistry Laboratory in Augusta, GA.

Hb A1c Analysis

Hb A1c was measured with a dedicated HPLC system (Hb A1c Analyzer, Model HA-8110; Daiichi, Kyoto, Japan). Erythrocyte hemolysates were prepared by mixing 3 μL of the EDTA blood sample with 450 μL of the hemolyzing reagent from Daiichi (5.6 mmol/L phosphate buffer with nonionic surfactant, pH 6.55), and incubated for 5 h at 37 °C to remove labile fractions of the glycated Hb. To separate the various Hb components, we used a column with a stationary phase of crosslinked spherical organic polymers (methacrylic acid and methacrylate copolymer; Micropearl SF-W-A1c, from Daiichi) that contained both ionic and hydrophobic groups for the ion-exchange reversed-phase chromatography. Chromatography was carried out at 22 °C at two different ionic strengths and pH values. The eluates were monitored at 415 and 500 nm.

Hemoglobin and Structural Analysis

Hematological data were obtained with an automated cell counter. The presence of an abnormal Hb was confirmed by cellulose gel electrophoresis and isoelectric focusing (11); the variants were quantified by cation-exchange HPLC with a PolyclA WXC column (PolyLC, Columbia, MD)(12). Structural analysis was carried out as previously described (13), with use of reversed-phase HPLC to separate the aberrant globin chain and HPLC systems to separate and purify the tryptic peptides. A Pico Tag amino acid analysis system (Waters, Milford, MA) was used to determine the amino acid composition of the isolated tryptic peptides.

Results

The two subjects were J.W., a 52-year-old Caucasian Canadian, and R.E., a 60-year-old Native Canadian Indian. Hb A1c analysis was carried out regularly to monitor their diabetic control. Figure 1 shows the Hb A1c chromatograms of the two patients. Further Hb analysis (data not shown) revealed a slow-moving Hb variant with an electrophoretic mobility just cathodic to Hb A at alkaline pH in J.W. and a slow-moving Hb variant with a mobility at the Hb G/D position in R.E. Chromatographic properties of these
two variants on the PolyCat column are shown in Figure 2.

Table 1 summarizes the quantitative analysis of the various Hb components and the hematological data of both patients. In J.W., the abnormal Hb composed 16% of the total Hb concentration. This result, coupled with the presence of an abnormal Hb A$\alpha$ (a$\alpha_6$A$\beta$) in the PolyCat chromatogram (Figure 2a), indicated that the abnormal Hb was an $\alpha$-chain variant. In patient R.E., the slow-moving abnormal Hb was 41.9% of the total Hb, indicating a possible $\beta$-chain variant. Other hematological data were unremarkable except for a slightly high concentration of Hb in J.W.

The abnormal $\alpha$-chain and abnormal $\beta$-chain were isolated by reversed-phase HPLC and CM-cellulose column chromatography, respectively, then aminoethylated and digested with trypsin. All normal tryptic peptides were recovered except for $\alpha$T-12 of J.W. and $\beta$T-3 of R.E. (data not shown). For J.W., two abnormal tryptic peptides were also recovered ($\alpha$T-12a and $\alpha$T-12b), whereas for R.E., an abnormal $\beta$T-3 was found. Table 2 lists the amino acid composition of two of the abnormal peptides. The abnormal $\alpha$T-12a with two Leu residues and one Arg residue was the result of the substitution of Arg for Ser in position 102 of the $\alpha$-chain. The Arg in position 102 created a new site for tryptic digestion, resulting in the $\alpha$T-12a tripeptide (Leu-Leu-Arg) at positions 100–102. Such a Ser$\rightarrow$Arg substitution at $\alpha$102 is consistent to that of Hb Manitoba, an abnormal Hb initially found in Canada (14).

The abnormal $\beta$T-3 differs from the normal $\beta$T-3 by an extra Ala residue and one less Glu residue; normal $\beta$T-3 has a Glu residue in positions 22 and 26. We assume that this substitution is in position 22, the same as that found in Hb G Coushatta, a common Hb variant found in North American Indians (15, 16).

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**Fig. 1.** Chromatographic separation and quantification of Hb $\alpha$T$\alpha$ in erythrocyte hemolysates of patients J.W. and R.E. with a Daichii HPLC Hb $\alpha$T$\alpha$ Analyzer

$X$: abnormal Hb peak

**Fig. 2.** Chromatographic separation and quantification of Hb components in erythrocyte hemolysates of patients J.W. and R.E. with a PolyCat-A HPLC column

$X$: abnormal Hb peak; $X_\alpha$, abnormal Hb $\alpha$ peak
Table 1. Hematologic and Quantitative Data from Two Diabetic Patients with Hb Manitoba and Hb G-Coushatta

<table>
<thead>
<tr>
<th></th>
<th>J.W.</th>
<th>R.E.</th>
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<tbody>
<tr>
<td>Hb variant</td>
<td>Hb Manitoba</td>
<td>Hb G-Coushatta</td>
</tr>
<tr>
<td>Hb, g/L</td>
<td>176</td>
<td>119</td>
</tr>
<tr>
<td>Packed cell vol, L/L</td>
<td>0.530</td>
<td>0.348</td>
</tr>
<tr>
<td>Erythrocytes, × 10¹²/L</td>
<td>6.0</td>
<td>3.74</td>
</tr>
<tr>
<td>Mean cell vol, fl</td>
<td>88</td>
<td>93.1</td>
</tr>
<tr>
<td>Mean cell Hb, pg</td>
<td>29</td>
<td>31.8</td>
</tr>
<tr>
<td>Mean cell Hb concn, g/L</td>
<td>335</td>
<td>342</td>
</tr>
<tr>
<td>Hb A₁, %</td>
<td>72.8</td>
<td>45.8</td>
</tr>
<tr>
<td>Hb A₂, %</td>
<td>2.1</td>
<td>3.1</td>
</tr>
<tr>
<td>Hb X, %</td>
<td>16.0</td>
<td>41.9</td>
</tr>
<tr>
<td>Hb Xₐ, %</td>
<td>&lt;1%</td>
<td></td>
</tr>
<tr>
<td>Hb F, %</td>
<td>0.7</td>
<td>0.5</td>
</tr>
<tr>
<td>Hb A₁₀, %</td>
<td>6.0</td>
<td>4.8</td>
</tr>
<tr>
<td>Total glycated Hb, %</td>
<td>8.2</td>
<td>10.5</td>
</tr>
</tbody>
</table>

* By PolyCat HPLC.
* By Betke’s alkaline denaturation.
* By the Dalichi Hb A₁₀ HPLC.
* Calculated as: %Hb A₁₀ divided by %Hb A and multiplied by 100.

Discussion

Over the last 15 years, Hb A₁c has proved to be a useful routine clinical laboratory procedure for evaluating long-term control of diabetes. Although many procedures have been developed for use, clinical laboratories must take into account that in some chromatographic methods the quantification of Hb A₁c may be inaccurate in the presence of abnormal Hb or of Hb F. Overestimation may occur in the presence of large quantities of Hb F, through incomplete resolution of Hb A₁c from Hb F, whereas underestimation may occur in the presence of abnormal Hb forms.

The first problem may be resolved by separately quantifying Hb F with procedures such as the Betke alkaline denaturation (17) and subtracting the amount of contaminating Hb F. The second problem requires the identification and quantification of the abnormal Hb (e.g., Hb S), followed by the addition of the glycated abnormal Hb to the glycated Hb A₁c to determine the total concentration of glycated Hb. For example, in the case of a sickle cell trait carrier (Hb A/S) with 70% Hb A and 30% Hb S, the Hb A₁c quantified by routine procedures reflects only 70% of the total glycated Hb. Similarly, in our patients, the Hb A₁c concentrations were underestimated because of the presence of the abnormal hemoglobins. The total glycated Hb can be calculated by dividing the Hb A₁c measurement by the percentage of Hb A times 100%. On the other hand, the use of the recently introduced “affinity columns,” which are supposed to determine the “total glycated hemoglobin,” may solve the problem of the presence of the common Hb variants.

The finding of two abnormal Hb variants during Hb A₁c analysis underscores the importance of evaluating the chromatographic patterns in routine Hb A₁c analyses. Any abnormal pattern needs to be repeated and further analyzed if the presence of an abnormal Hb is suspected. Once the proportions of Hb A and the abnormal Hb are known, the actual total glycated Hb can be determined by adding the two.

Interestingly, the two abnormal Hb variants we found are common in Canada. Hb Manitoba is an α-chain variant that was initially found in a Canadian family of British origin (14); it has also been found in another Canadian family of German–Irish origin (15). J.W. is a Native Canadian with some Scottish descent. Hb G-Coushatta is a β-chain variant commonly found in Native Canadian Indians (19, 20); our patient, R.E., is a Native Canadian Indian living in the Province of Alberta. The structural abnormalities in these two Hb variants do not directly involve the crucial heme contacts or α₁β₂ contacts that are important for the stability and oxygenation–deoxygenation process of the Hb molecule. The mild anemia and slight erythrocytosis in these patients are probably secondary and are not related to the hemoglobinopathy.

We thank Drs. S. Ross and N. Campbell for the blood samples for this study. We also thank Mr. J. B. Wilson and Professor T. H. J. Huisman of the Medical College of Georgia for the structural identification of the Hb variants.

References

Table 2. Amino Acid Composition and Sequence of the Abnormal Tryptic Peptides in Two Diabetic Patients with Hb Manitoba and Hb G-Coushatta

<table>
<thead>
<tr>
<th>Peptide</th>
<th>J.W.</th>
<th>R.E.</th>
</tr>
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<tbody>
<tr>
<td>Amino acid position</td>
<td>αT-12a</td>
<td>βT-3</td>
</tr>
<tr>
<td>Normal amino acid residues</td>
<td>100 to 102</td>
<td>18 to 30</td>
</tr>
<tr>
<td>Recovered amino acid residues</td>
<td>2 Leu, 1 Ser</td>
<td>2 Asp, 2 Glu, 3 Gly, 1 Arg, 1 Ala, 3 Val, 1 Leu</td>
</tr>
<tr>
<td>Normal amino acid sequence</td>
<td>2 Leu, 1 Arg</td>
<td>2 Asp, 1 Glu, 3 Gly, 1 Arg, 2 Ala, 3 Val, 1 Leu</td>
</tr>
<tr>
<td>Abnormal amino acid sequence</td>
<td>Leu-Leu-Ser</td>
<td>Val-Aan-Val-Asp-Glu-Val-Gly-Gly-Glu-Ala-Leu-Gly-Arg</td>
</tr>
</tbody>
</table>

Changes from normal composition are underlined.