Increased Fetal Hemoglobin in Insulin-Treated Diabetes Mellitus Contributes to the Imprecision of Glycohemoglobin Measurements

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An increased prevalence of fetal hemoglobin (HbF) has been described in pediatric insulin-dependent patients. The popular electroendosmotic method for glycohemoglobin includes HbF. In an adult population comprising 50 insulin-treated and 57 non-insulin-treated diabetic patients and 57 control subjects, we measured HbF by HPLC and measured glycohemoglobin by both HPLC and an electroendosmotic method. Of the insulin-treated patients, 46% had concentrations of HbF ≥0.5%, compared with 25% of non-insulin-treated patients and 23% of controls (P <0.02). In the insulin-treated patients, the two glycohemoglobin methods correlated best when the HPLC measurements included HbF (r = 0.92 vs r = 0.84). Fructosamine concentrations correlated best with glycohemoglobin concentrations determined by methods that accounted for HbF. The true between-batch CV of the electroendosmotic assay increased (from 4.33% to 8.33%) when variable interpatient HbF concentrations were included. Thus, HbF must be considered when interpreting glycohemoglobin measured by an electroendosmotic method and when comparing it with other measures of glycemic control.

Indexing Terms: hemoglobin variants • electroendosmosis • chromatography, ion-exchange • variation, source of

Glycohemoglobin (HbA1) has gained widespread use as an indicator of time-integrated glycemia over the 4–6 weeks preceding its measurement (1). A popular method of measuring HbA1c is by electroendosmosis (2, 3). This method, however, includes fetal hemoglobin (HbF), both glycated and nonglycated, which comigrates in the measurement and so may lead to spuriously high determinations of HbA1c (4).

An increased prevalence of HbF in pediatric Type I (insulin-dependent) diabetic patients compared with that in nondiabetic controls has been described (5). Here we describe the prevalence of HbF in insulin-treated and non-insulin-treated diabetic adults compared with a group of nondiabetic controls. We also studied the effect that HbF concentrations may have on HbA1c measurement by electroendosmosis.

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Patients and Methods

Diabetic patients were chosen without conscious bias from the outpatient clinic at Gartnavel General Hospital, Glasgow: 50 were insulin-treated (27 men, 23 women; median age 40, range 13–77 years) and 57 were non-insulin-treated (31 men, 26 women; median age 64, range 36–94 years). Also included in the study were 57 nondiabetic control patients (34 men, 23 women; median age 59, range 12–90 years). Consent for venesection was obtained from all participants.

Whole blood was collected in test tubes containing K+ EDTA for the measurement of HbA1c, HbA1, and HbF, the samples being analyzed within 2 days. Serum was separated for fructosamine analysis in 82 of the patients.

HbA1c was measured by electroendosmosis (HbA1c-E) and high-performance liquid chromatography (HbA1c-HPLC) methods.

In measurement by electroendosmosis (Ciba Corning Diagnostics, Ltd., Halstead, Essex, UK), the sample (50 μL) was incubated at 37 °C with 150 μL of labile-fraction-removing hemadelaying reagent. Hemolysate (1 μL) was dispensed onto an agar film and underwent electroendosmosis in a citrate buffer. The film was then dried and scanned at 420 nm by a Corning 720 densitometer, which displayed the result for HbA1c as a percentage of the total hemoglobin. Between-batch imprecision (CV) was 4.33% at a mean HbA1c concentration of 8.22% (n = 107).

For the HPLC method (Hi-AutoA1c, Model 8121; Kyoto Daiichi Kagakui Co. Ltd., Kyoto, Japan), 3 μL of whole blood was added to 450 μL of hemolyzing solution containing a labile-fraction-removing reagent (tetrapolyphosphate) and incubated at 48 °C for 2 min at pH 6. The HPLC ion-exchange chromatographic column yielded a separation time of 4 min. The effluent was monitored by a dual-wavelength photometer and measurements of HbA1c, HbA1c-HPLC, and HbF were obtained by the integration of their corresponding peaks. The concentrations of HbA1c, HbA1c, and HbF were expressed as a percentage of the total hemoglobin. Within-batch imprecision for HbA1c-HPLC was 1.39% at a mean HbA1c concentration of 4.91%. For HbA1c-HPLC the CV was 1.37% at a mean concentration of 6.56%. At mean HbF concentrations of 0.405% and 0.890%, the CVs were 5.5% and 3.45%, respectively.

Plasma glucose was measured by a glucose oxidase method (Beckman Instruments Inc., High Wycombe, Bucks, UK). Between-batch imprecision (CV) at a mean glucose concentration of 5.24 mmol/L was 2.7%.

Serum fructosamine was determined by a nitroblue
tetrazolium reduction method (Fructosamine Plus; Roche Diagnostics, Welwyn Garden City, Herts, UK) with a Cobas-Bio centrifugal analyzer (Roche Diagnostics). Between-batch imprecision was 1.90% at a mean concentration of 304.7 μmol/L.

For statistical analysis we used Kruskal–Wallis one-way analysis of variance and chi-squared tests with Yates correction where appropriate. Correlation coefficients were calculated by the least-squares method. Statgraphics software (Statistical Graphics Corp., Rockville, MD; 1986 version) was used throughout.

Results

Insulin-treated diabetic patients had a mean HbF concentration of 0.40% (range 0–2.7%) and non-insulin-treated diabetic patients 0.30% (range 0–4.7%) (P = 0.056, by Kruskal–Wallis). Control patients had a mean HbF concentration of 0.34% (range 0–3.1%), not significantly different from the diabetic group.

Increased proportions of HbF are those ≥0.5% of total hemoglobin (5). Twenty-three of 50 (46%) insulin-treated patients had increased concentrations of HbF, compared with 13 of 57 (23%) control subjects (P < 0.02, chi-squared test). Fourteen of 57 (25%) non-insulin-treated diabetic patients had increased HbF concentrations (not significantly different from the control subjects).

HbF concentrations did not correlate with the measures of diabetic control represented by HbA\(_{1\text{E}}\), HbA\(_{1\text{C}}\), or fructosamine.

Age and sex had no significant effect on the prevalence of increased HbF concentrations in insulin-treated, non-insulin-treated, or control groups (Kruskal–Wallis test).

HbA\(_{1\text{E}}\) correlated with HbA\(_{1\text{C}}\)-HPLC in insulin-treated patients (r = 0.84, P < 0.001) and in non-insulin-treated patients (r = 0.93, P < 0.001). The correlations improved when HbA\(_{1\text{E}}\) (in which HbF comigrates with HbA\(_1\)) was compared with the sum of HbF\(_{\text{HPLC}}\) and HbA\(_{1\text{C}}\)-HPLC (r = 0.92 and 0.95, respectively).

Serum fructosamine correlated best with HbA\(_{1\text{C}}\)-HPLC (r = 0.76, P < 0.001), followed by HbA\(_{1\text{C}}\)-HPLC (r = 0.72, P < 0.001) and (HbA\(_1\) + HbF\(_{\text{HPLC}}\) (r = 0.68, P < 0.001).

Fructosamine correlated least with HbA\(_{1\text{E}}\) (r = 0.64, P < 0.001).

To illustrate the possible effect of HbF on the precision of the electroendosmotic assay, we used HbF-free quality-control material. The estimated imprecision (CV) of determinations of this preparation was 4.3% at a mean HbA\(_1\) concentration of 8.22% (Figure 1). Patients' samples, however, contain various concentrations of HbF, which are included in HbA\(_{1\text{E}}\) measurements. To simulate the effect that variations in patients' HbF content might have on assay imprecision, we added in no set order the concentrations of HbF\(_{\text{HPLC}}\) measured in 107 diabetic patients to the previous 107 electroendosmotic quality-control results. The mean calculated HbA\(_1\) concentration increased to 8.57% and the CV to 8.33% (Figure 1).

Discussion

HbF is well known to comigrate with HbA\(_1\) in electroendosmotic measurements of HbA\(_1\). Ion-exchange chromatography also exhibits this phenomenon (4).

HbF concentrations do not appear dependent on age, sex, or degree of glycemic control, but the results of the present study suggest that insulin-treated patients may have greater values than do non-insulin-treated and nondiabetic control subjects. In a pediatric population the HbF concentration was demonstrated to be greater in patients with insulin-dependent diabetes than in control subjects; however, the concentrations declined into adulthood (5). Our observations suggest that this may not be the case in some patients.

Increased proportions of HbF have been described in autoimmune diseases such as pernicious anemia (6) and thyrotoxicosis (7). Because Type 1 (insulin-dependent) diabetes mellitus has an autoimmune component (8), it is perhaps not surprising that increases in HbF concentrations may also be present.

The reason for increased HbF concentrations remains...
speculative. A delayed transition from HbF to adult HbA has been described in the hyperinsulinemic infants of diabetic mothers (9), but with abnormal HbF concentrations appearing unrelated to age in our study, we think a reactivation of the HbF gene in insulin-treated patients seems more probable.

Increasing reliance is being placed on treating patients on the basis of their HbA1 result. The European Non-Insulin-Dependent Diabetes Policy Group has issued recommendations regarding the classification of glycosylated control as reflected in glycohemoglobin measurements (10). These define good control as a HbA1 measurement <2 SD from the mean for the non-diabetic population, i.e., within the reference range. Control is acceptable within 4 SDs, but is regarded as poor outside this range. In our laboratory, these values correspond to electroendosmotic HbA1 measurements of <7.8% (good), ≤9.3% (acceptable), and >9.3% (poor). As we have demonstrated, the concentrations of HbF not only affect those patients with high HbF measurements (and therefore spuriously high HbA1) but also have a general effect on the imprecision and accuracy of the electroendosmotic assay.

If all patients had the same concentration of HbA1 and HbF, then only the assay accuracy, not imprecision, would differ from that derived from a control value. However, because interpatient HbF concentrations vary, so does the accuracy for each patient. This variation in accuracy of patients' samples must therefore add to the imprecision measured by a control solution.

If 2 SD from a quality-control mean is regarded as acceptable when an assay is performed, then, by our simulated experiment, a patient with a HbA1 of 8.22% may actually have an electroendosmotic measurement between 7.14% and 10.00% (Figure 1). By the above definition, this corresponds to either good, acceptable, or poor glycosylated control.

The comparison between HbA1 measured by electroendosmotic and by other methods (which do not include fetal hemoglobin) is influenced by HbF concentrations in patients' samples. In this study, in which we compared the electroendosmotic measurement with HPLC, the correlation between HbA1 measurements in insulin-treated patients was less (r = 0.84) than when the measured HbF concentration was included (r = 0.92). Thus, in laboratories using the electroendosmotic method of HbA1 measurement, the potential effect of HbF concentrations should be taken into account when comparing different methods of analysis.

Our previous work has indicated that parallel measurements of fructosamine and glycohemoglobin add to the information obtained by the use of either method alone (11). Comparisons between fructosamine and HbA1, as different assessments of glycemic control have shown wide variations in the correlation between the two measurements, varying from r = 0.91 (12) to a complete lack of correlation (13). The discrepancy in the time period of integrated glycosia measured by the two assessments (1–3 weeks for fructosamine, 4–6 weeks for HbA1) is likely to be partially responsible, especially during periods of changes in diabetic control (11).

An important finding in the present study is that comparisons differ markedly with the method of HbA1 measurement and that inclusion of HbF accounts for some of the discrepancies. Correlation between fructosamine and HbA1 was least for the electroendosmosis method (r = 0.64). For HPLC, the correlation was least when including measured HbF, (HbA1 + HbF), but improved when measuring just HbA1-HPLC (r = 0.68 and 0.72, respectively). Correlation with fructosamine was greatest with HbA1c-HPLC (r = 0.76). Thus future comparisons of the two assessments may benefit if HbA1c rather than total HbA1 is measured.

Our findings suggest that the popular electroendosmotic method of HbA1 measurement may be less accurate and precise than the quality-control data suggest, especially in insulin-treated patients. Thus the increasing use of other methods (e.g., HPLC, immunoassays with monoclonal antibodies to HbA1c) appears justified—if these methods can improve the reliability of glycohemoglobin measurements and therefore ultimately lead to a more dependable assessment of glycoemic control.

We acknowledge the analytical help of S. Cunningham in the preparation of this article.

References