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Review Articles

Haemoglobin A1 and Diabetes Mellitus

B. Gonen and A. H. Rubenstein

Department of Medicine, University of Chicago, Chicago, Illinois, USA

Summary. $HbA1_c$ is the product of a reaction between glucose and the N-terminal valine of adult haemoglobin (HbA). The levels of $HbA1_c$ are increased in diabetic patients, and reflect their metabolic control. Thus, measurements of $HbA1_c$ have proved to be useful in the follow up and treatment of diabetic patients. $HbA1_c$ may prove to be valuable for assessing the relationship between diabetic control and long-term complications, as well as in studying the potential glycosylation of proteins in various organs which may occur in the diabetic state.

Key words: Haemoglobin $A1_c$, glycosylated haemoglobins, diabetic control, diabetic complications.

Despite the increase in our understanding of the pathogenesis and therapy of diabetes mellitus, an important question remains mostly unresolved. It concerns the relationship between the degree of metabolic control and the occurrence of long-term diabetic complications. While many studies suggest that certain complications can be prevented or delayed by improving metabolic control in diabetic patients, there is still considerable controversy as to the energy and effort that should be spent, both by physicians and patients, in the pursuit of normoglycaemia, particularly if it is at the expense of worsening hypoglycaemic reactions [1–3].

One of the main reasons for the difficulty in resolving this problem is the lack of a satisfactory method for the quantitative assessment of diabetic control. Such a method would be helpful not only for conducting long term studies on the cause and effect relationship between diabetic control and late complications, but also, hopefully, for the day to day management of diabetic patients. There is little doubt that most of the present methods for determining the degree of metabolic control, though useful, are either inadequate (random blood sugar estimations, intermittent measurements of daily urinary glucose excretion), unreliable (urine fractional collections performed at home by the patient) or cumbersome and impractical for application in an outpatient setting (continuous 24 hour blood glucose monitoring). It is in the light of these problems that the development of a new test, haemoglobin $A1_c$, as an indicator of the integrated plasma glucose level over prolonged periods of time has recently aroused interest (4–6).

Chemistry

In most adults the major form of haemoglobin in red cells is haemoglobin A (or AII)[1], which comprises more than 90% of the total haemoglobin concentration. Its subunit structure is $\alpha_2\beta_2$. The remaining 10% of the haemoglobin is made up of $HbA1_c$, which comprises 4–6%, $HbA1_a$ and $HbA1_b$, each comprising approximately 1–2%, and possibly other minor components, $HbA1_d$ and $HbA1_e$. The term HbA1 denotes the sum of all these minor haemoglobins.

The minor haemoglobins can be separated from HbA and one another by various chromatographic methods [7, 8]. At a slightly acidic pH (6.7) the minor haemoglobins are more negatively charged than HbA. Thus, if a haemolysate is applied to a cation exchange resin and eluted with a buffer, pH 6.7, $HbA1_a$ will emerge first, followed by fractions containing $HbA1_b$ and $HbA1_c$. HbA is bound to the

[1] This major form of haemoglobin has also been called A_o by other investigators.

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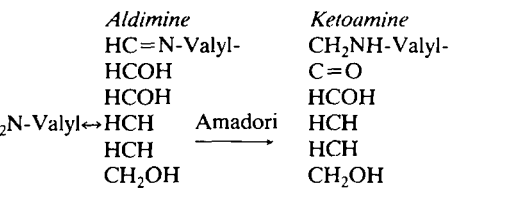

Fig. 1. Reaction between glucose and valine at the amino-terminal end of the beta chain of haemoglobin A

resin and can be eluted by changing the pH and ionic strength of the buffer. The specific methodology for quantitating the minor haemoglobins will be discussed in more detail at the end of this section.

$HbA1_c$ was first isolated by Allen et al. [7]. Subsequently it was appreciated that $HbA1_c$ was structurally identical to HbA, except for the presence of an additional negatively charged molecule. The position of this negatively charged group was localized to the N-terminal end of the β-chain [9]. Several investigators attempted to identify the substance responsible for the negative charge on the $HbA1_c$ molecule, and in 1975, Bunn et al. established that a glucose molecule was bound to the N-terminal valine residue by a Schiff's base [10]. Upon treatment of the NH_2-terminal glycovalylhistidine isolated from the β-chain with sodium borohydride, glucitol and mannitol-valines are formed. This finding implies that 1-deoxy-1-(N-valyl) fructose is the actual common intermediate bound to the amino-terminus of the β-chain in $HbA1_c$ [11]. The stability of the aldimine linkage is markedly increased by an Amadori rearrangement to form a ketoamine bond [10]. This chemical modification is illustrated in Figure 1.

There is convincing evidence that the glycosylation of HbA to form $HbA1_c$ occurs in the red blood cell in the peripheral circulation during its entire life span. Bunn et al. [12] have infused ^{59}Fe-labelled-transferrin into two individuals. The specific activity of HbA reached its maximum within a week, when a plateau was achieved, whereas the specific activity of $HbA1_c$ and the other minor haemoglobins continued to rise slowly until the sixtieth day. Fitzgibbons et al. [13] have shown that the $HbA1_c$ content of old erythrocytes was significantly higher than the content of young erythrocytes in cells derived from both healthy subjects and diabetic patients. Furthermore, the level of $HbA1_c$ is reduced in patients whose red blood cells have a shortened life span due to haemolysis [12]. This slow rate of posttranscriptional glycosylation is characteristic of a non-enzymatic reaction, is probably irreversible and is mainly dependent upon the prevailing blood glucose concentration.

Whether glucose reacts directly with the NH_2-terminal valine, or an intermediate is involved, is at present unknown. Haney and Bunn [14] have provided evidence in favour of the latter suggestion. These investigators demonstrated that [^{14}C]-glucose 6-phosphate binds to HbA in vitro at a much faster rate than does ^{14}C-glucose, suggesting that glucose-6-phosphate may be an obligatory intermediate in the formation of $HbA1_c$. While most of the bound [^{14}C]-glucose eluted in the fraction corresponding to $HbA1_c$, some [^{14}C]-glucose also emerged in the HbA fraction. This finding indicates that glucose can bind non-specifically and reversibly to HbA at a site(s) other than the NH_2-terminal β-chain, and that these compounds are not separated from HbA by the currently employed chromatographic methods (45, 46). In contrast to [^{14}C]-glucose, [^{14}C]-glucose-6-phosphate did not bind non-specifically to HbA. These studies and others [15] demonstrate clearly that $HbA1_c$ can be formed in vitro by adding glucose solutions to HbA, though the reaction of HbA with phosphorylated hexoses occurs at a faster rate [16]. The glycosylation reaction is inhibited by 2,3-diphosphoglycerate (2,3-DPG), carbon monoxide or nitrogen [12]. It is of interest that glycosylation of HbS to form $HbS1_c$ may also occur in the circulation [14]. In this regard, the possibility of reducing sickling by glycosylation of HbS has been raised, but this idea has not yet been tested.

The structures of the two other minor haemoglobins, $HbA1_a$ and $HbA1_b$ are less certain. Using improved chromatographic techniques, McDonald et al. [17] have shown that $HbA1_a$ can be separated into two components: $(Hb)A1a_1$ and $(Hb)A1a_2$. Analyses of the glucose and phosphate content of these molecules, as well as preparation of synthetic glucohaemoglobins has led to the conclusion that the molecule present at the NH_2-terminus of the β-chain of $HbA1a_1$ is fructose 1,6-diphosphate, while in $HbA1a_2$ it is glucose-6-phosphate. The nature of $HbA1_b$ is unknown. The possibility that $HbA1_a$ or $HbA1_b$ may be precursors of $HbA1_c$ has also been suggested [14, 16], but more recent evidence indicates that this is not so [17].

When it was appreciated that glycosylation of HbA occurred in vivo, it stimulated speculation that glycosylation of other proteins may also occur. For instance, glucose may bind to proteins in basement membranes of various organs and contribute to the morphological alterations which have been described in long-standing diabetics. Evidence suggesting that these changes may in fact take place is derived from studies of the structure of the renal-glomerular basement membrane. This material has an increased content of disaccharide and heteropolysaccharide molecules [18] in diabetic animals. If this finding proves to be a feature of other proteins, it will

undoubtedly stimulate further research into the regulation of the biosynthetic pathway of glycosylated proteins, as well as studies of pharmacological agents capable of inhibiting the glycosylation reaction or of removing the sugar moiety from these proteins. This line of investigation has great clinical potential.

Methodology

The methodology currently in use in most laboratories to measure HbA1 is based on the principles outlined in the previous section, namely, chromatography on cation exchange columns. The columns that were originally used were relatively long (approximately 30 cm) and the elution time required many hours. Shortening of the column to 8–10 cm permitted the assay to be performed in two hours and significantly increased the number of tests which a technician could handle each day [16]. The method currently in use in our laboratory involves the following steps: 6–8 ml blood is placed in a tube containing EDTA at 4° C. The plasma is removed and the red cells are washed three times with 0.9% NaCl; the cells are then haemolysed with distilled water and toluene added to separate the membranes. After centrifugation, the watery layer is incubated overnight in the cold with developer 10 x buffer, containing KCN, to convert methaemoglobin to cyanmethaemoglobin (haemolysate). On the day of assay, 100 or 150 µl of this haemolysate is placed on a cation exchange resin column equilibrated in developer 1x buffer. The optical density of the eluate is monitored in a spectrophotometer at a wavelength of 421λ. Following elution of the first peak containing the fast haemoglobins, the buffer is changed to one with a high phosphate concentration and the major haemoglobin component, HbA, is then eluted. The ratio of the haemoglobin concentration (calculated as O. D. x fraction volume x extinction coefficient) in the first fraction to the sum of concentrations in the first and second fractions is calculated and the HbA1 result expressed as a percentage of the total haemoglobin. A detailed description of this method can be found in the paper by Gabbay et al. [19] and is based on a modification of the method described by Trivelli et al. [20].

The separation of the minor haemoglobins from each other is improved by using longer columns. Nevertheless, because the separate measurement of $HbA1_c$ probably does not offer any special advantage in the clinical setting, most laboratories measure the sum of the minor haemoglobins, $HbA1_a$ + $HbA1_b$ + $HbA1_c$ (HbA1). With the modified method using short columns (19–21), the number of samples which can be measured in a day by a single technician is still limited to 30–40. A new approach to the measurement of the glycosylated haemoglobins is the use of high pressure liquid chromatography. The preliminary results which have been reported [22, 23] are encouraging. The time required for the assay of a single sample with this method is only a few minutes. Further information is now awaited on the use of this method in the study of diabetic patients.

Clinical Studies

The association of increased $HbA1_c$ levels with diabetes mellitus was first noted by Huisman and Dozy (24) and later confirmed and extended by Rahbar and his colleagues [25, 26]. In a large survey in Teheran, Rahbar detected an abnormal haemoglobin band in 2 of 1200 people tested. On further inquiry both of these individuals proved to be diabetic. Moreover, this haemoglobin band was present in the blood of every diabetic patient that he subsequently examined. It also became clear that this minor haemoglobin had indentical chromatographic and electrophoretic properties to $HbA1_c$ which had been identified by then in small quantities in healthy subjects [8, 9]. Thereafter, efforts were concentrated on elucidating the structure of this haemoglobin molecule and measuring its concentration in different patient populations.

One of the first clinical studies of $HbA1_c$ levels in diabetic patients was reported by Trivelli et al. [20]. Their results were expressed as total HbA1, which represents the combined sum of the three minor fast-running haemoglobins. The mean (± SD) HbA1 value for control subjects was 6.5±1.5%, whereas the values for adult onset and juvenile onset diabetics were 11.1±2.9% and 12.4±3.1% respectively [20]. Similar results have subsequently been reported in many other studies [19, 27–29]. The distribution of HbA1 levels among our population of diabetic patients is shown in Figure 2. The highest levels of HbA1 were found in diabetic patients during episodes of keto-acidosis and severe infection, when they were obviously in poor metabolic control. On the other hand, the HbA1 concentrations were not related to any of the following: age of the patient, mode of treatment, duration of diabetes or presence of renal failure, peripheral vascular disease, retinopathy or neuropathy.

Stimulated by the findings of Trivelli et al. [20] clinical research proceeded in the following main directions:

I. $HbA1_c$ was measured in different disease states associated with either pancreatic dysfunction or structural abnormalities of the erythrocyte.

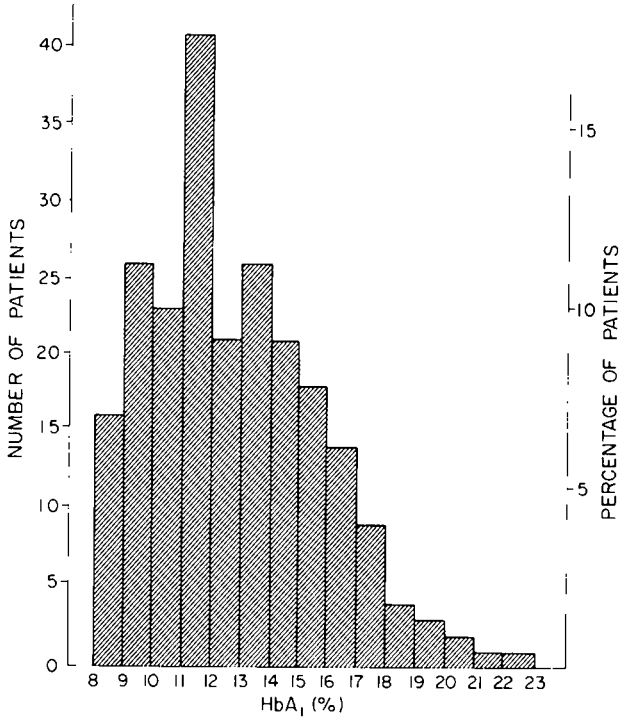

Fig. 2. Distribution of HbA1 levels among 227 diabetic patients

II. In patients with diabetes mellitus:

a) $HbA1_c$ was measured at various stages of the disease and in different groups of patients.

b) The effect of increased levels of $HbA1_c$ on the function of red blood cells was assessed.

c) $HbA1_c$ (or HbA1) was evaluated with regard to its:

1) relationship to some of the metabolic and hormonal derangements present in diabetes (such as in lipid metabolism, glucagon and growth hormone concentrations);

2) association with short and long term diabetic complications;

3) relationship to various derived parameters of plasma glucose concentrations. This approach was carried out in an attempt to define its usefulness as an indicator of metabolic control and its application to the clinical care of diabetic patients.

I. HbA1 in Diseases Other Than Diabetes Mellitus

HbA1 levels were elevated in infants with cystic fibrosis, who had low insulin reserve but normal glucose tolerance tests [27]. In conditions where there is a shortened life span of the erythrocyte, the levels of the glycosylated haemoglobins were low [12], and in diseases in which abnormal haemoglobins exist (e. g. thalassaemia) the levels "HbA1" are high with the methods of assay presently in common use. Obviously HbA1 cannot be used as an indicator of diabetic control in these patients. Increased levels of $HbA1_c$ have also been reported in uraemic patients who are undergoing chronic haemodialysis [30]. It is not clear whether this is only the result of the high glucose content of the dialysate fluid.

II. HbA1 in Diabetes Mellitus

a) HbA1 in Different Stages and Populations of Diabetic Patients: In prediabetics (both parents diabetic or an identical diabetic twin) the levels of HbA1 were within the normal range [29]. This finding is consistent with the postulate that abnormal levels of HbA1 are secondary to the metabolic abnormality of diabetes, rather than being genetically determined.

Diabetic keto-acidosis was associated with very high levels of glycosylated haemoglobins, probably reflecting the prolonged period of poor control preceding the acute episode [20, 27]. In patients with diabetes secondary to Cushing's syndrome, the glycosylation reaction occurs at the same rate as in idiopathic diabetes, resulting in $HbA1_c$ levels comparable to those found in these latter patients [27].

Total HbA1 was within the normal range in pregnant women [27]. When $HbA1_c$ was separated from the other minor haemoglobins, its levels in normal pregnancy were increased ($6.97 \pm 0.69\%$ compared to $5.74 \pm 0.42\%$ in non-pregnant females) even though the authors did correct for the contribution of HbF [31]. In patients with gestational diabetes $HbA1_c$ levels were significantly raised [31]. However, the levels of $HbA1_c$ were lower in the pregnant diabetic patients compared to the values in non-pregnant diabetic women. The authors' speculation that a circulating factor which inhibits glycosylation is present during pregnancy may be unnecessary, because they did not show the results of their patients' glucose levels. Thus the degree of control may have been different in the two groups.

In healthy children, the levels of HbA1 were comparable to those in adults [32].

b) HbA1 and Erythrocyte Function: Doubling or even tripling of the $HbA1_c$ content of the erythrocyte, as frequently occurs in diabetic patients, may compromise the oxygen carrying capacity of these cells [33–35, 44]. Ditzel [33] has shown that the P_{50} at the in vivo pH of whole blood of ambulatory, non-acidotic diabetic patients is significantly lower than normal. This decrease occurs because the binding of 2,3-DPG to $HbA1_c$ is inhibited by the additional glucose molecule linked to the amino-terminal end of the β-chain. Ditzel and Standl [35] have suggested that this increase in oxygen affinity is responsible for

tissue hypoxia, which, in the face of inadequate compensatory mechanisms, leads to changes characteristic of the long term microvascular complications of diabetes.

c) HbA1, Diabetic Control and Long Term Complications: Peterson et al. [36] have shown that as glucose levels improved in diabetic patients there was a concomitant decrease in plasma triglyceride concentrations which correlated well with the falling HbA1 levels. The changes in cholesterol concentrations correlated less well with HbA1. In another study [37], a small but statistically significant correlation between the fasting levels of triglycerides and HbA1 was observed: however, this correlation was only present if patients with extremely high triglyceride levels were excluded. Gabbay and his co-workers [19] have demonstrated a significant correlation between fasting cholesterol and HbA1 levels. It should be noted that the r values for the correlations between HbA1 and cholesterol and HbA1 and triglycerides are low, implying that the cholesterol and triglyceride concentrations are affected by other factors in addition to the degree of diabetic control.

The potential use of HbA1 measurements in studies designed to determine whether long term diabetic complications are a consequence of less than optimal metabolic control is of great importance. At this time there are no published results of prospective studies which address this question. Trivelli et al. [20] did not find a difference in the HbA1 levels of diabetic patients with or without complications. There was no correlation between HbA1 levels and the thickness of skeletal muscle capillary basement membrane in a group of 21 diabetic patients [38]. Peterson et al. [39] have hospitalized poorly controlled diabetic patients and measured their $HbA1_c$ levels as well as leucocyte and platelet function at frequent intervals for two months. As the metabolic control improved, $HbA1_c$ levels decreased and the length of the secondary lag phase of platelet aggregation following epinephrine stimulation increased significantly. The authors concluded that $HbA1_c$ levels correlated with this diabetes-induced short term defect of platelet function and that the abnormality was reversible when metabolic control improved.

The rate of glycosylation of haemoglobin A to form $HbA1_c$ is thought to be predominantly determined by the prevailing plasma glucose levels. It is therefore not surprising that the levels of HbA1 correlate well with various parameters derived from multiple glucose measurements carried out at different times during the day in diabetic patients. However, the excellence of this correlation was somewhat unexpected. Graf et al. [40] have reported that a correlation exists in normal subjects between their fasting plasma glucose levels and HbA1 concentrations. The authors suggested that this correlation is best expressed as a curvilinear function. If this relationship between the blood glucose and HbA1 can be extended to diabetic patients, one would anticipate that as normoglycaemia is approached, any given change in glucose concentration would be associated with a greater change in the HbA1 value. This finding may explain our clinical experience that the HbA1 of most diabetic patients can be lowered to approximately 10% with relative ease, but further improvement is extremely difficult to achieve.

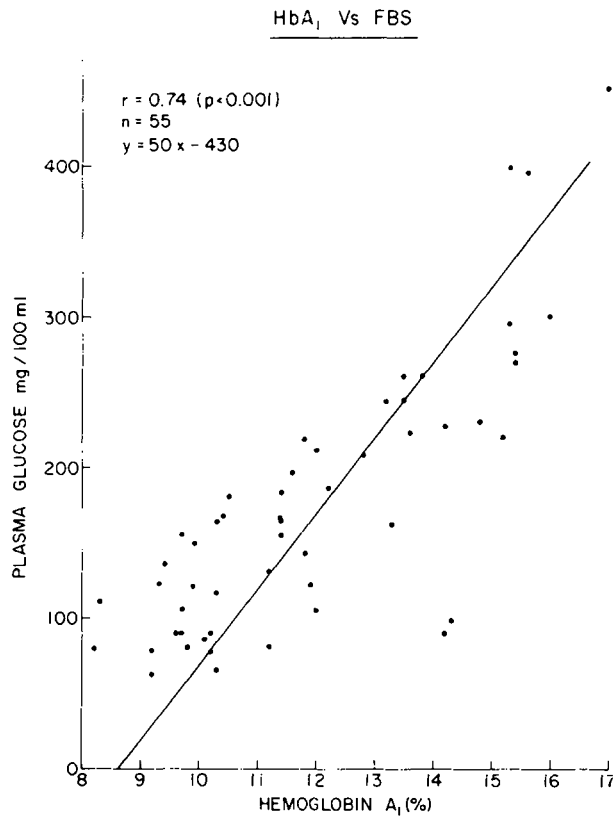

Fig. 3. Correlation between fasting glucose levels and HbA1 concentrations in 55 diabetic patients. (Reproduced from [28] with permission)

The relationship of HbA1 to the fasting plasma glucose concentration (Fig. 3), mean daily plasma glucose value (calculated from frequent glucose determinations over a 24 hour period) and highest plasma glucose concentration attained during 12 hours of continuous glucose monitoring has been assessed [28]. The result showed a significant correlation between HbA1 levels and all these parameters. In the same study, physicians rated the degree of diabetic control of their patients on a scale from 1–5. A rating of 1 was defined as the poorest control, 5 represented the best control and 2, 3, 4 were defined

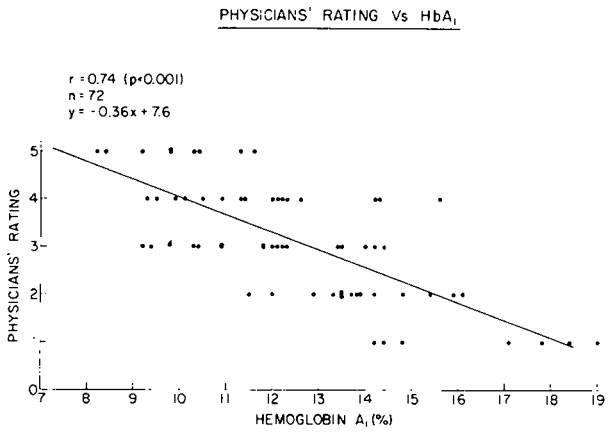

Fig. 4. Correlation between the rating of the degree of metabolic control and HbA1 concentrations in 72 diabetic patients (see text for explanation). (Reproduced from [28] with permission)

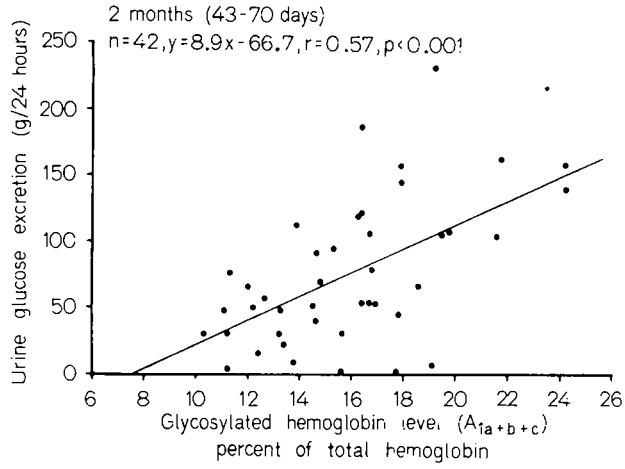

Fig. 5. Correlation between urine glucose concentrations and HbA1 levels in 42 diabetic patients. (Reproduced from [19] with permission)

as poor, fair and good control, respectively. The correlation between HbA1 values and the rating of metabolic control as evaluated by the physicians was highly significant (Fig. 4).

Gabbay et al. [19] found a significant correlation between HbA1 levels and 24 hour urinary glucose excretion which was measured at monthly intervals in 128 diabetic patients. The best correlation was noted between the level of HbA1 and the urinary glucose excretion measured two months previously ($r = 0.57$; $p < 0.001$; Fig. 5). In this regard it is of interest that 60 days represents the normal half-life of circulating erythrocytes. In a recent study, a correlation between $HbA1_c$ and daily fractional urinary glucose estimations was found in a small group of diabetic patients [41].

Koenig et al. [42] hospitalized five diabetic patients and adjusted their insulin therapy and diet so as to markedly improve their control. Glucose levels, which were measured as "brackets" (sum of pre-and post-meal levels, three times a day), decreased and the numerical values of the "brackets" correlated significantly with $HbA1_c$ concentrations. The fall in $HbA1_c$ levels followed the decrease in glucose "brackets" (and 24 hour urinary glucose excretion) by 3 to 4 weeks. This lag period was also noted in animals by Koenig and Cerami [43] who showed a four weeks delay from the time of onset of hyperglycaemia in genetically diabetic mice and the ensuing increase in $HbA1_c$ levels.

These results indicate why the possibility of using the HbA1 concentration as an indicator of diabetic control is so intriguing. The shortcomings of present methods for assessing the metabolic control in diabetic patients are well known. Testing the glucose concentration of urine samples at frequent intervals is the most widely used method and decisions regarding changes in insulin dosage and dietary intake are often based on these results. However, it is both demanding and time consuming for the patient and is unreliable in the presence of renal disease. Quantitation of 24 hour urinary glucose excretion is also inaccurate in patients with renal disease and is prone to serious errors in urine collection. Fasting or postprandial plasma determinations represent only one point in time and need to be repeated frequently in order to obtain an overall picture of metabolic control. The reproducibility of blood glucose estimations depends upon the time of day when the sample is drawn, content of the previous meal and activity of the patient, as well as the inherent instability of the diabetic state. In many instances widely disparate values are obtained at different times.

An additional problem in assessing diabetic control is the lack of agreement as to the blood glucose concentrations which are optimal and attainable [1]. The definitions vary from "normal" plasma glucose levels at all time, to less than 5% of carbohydrate intake excreted in the urine over a 24 hour period or to "negative" or "trace" glycosuria in multiple fractional urinalyses. It seems probable that the measurement of HbA1 will serve as a standardised method for describing metabolic control in diabetic patients. Thus, a patient whose HbA1 level is 12% can be considered to have been in better control than another patient whose value is 14%, but less well controlled than a patient with a HbA1 level of 10%. This is in marked contrast to the situation in which three patients present with fasting glucose levels of 200, 220 and 180 mg%, respectively, or with 24 hour urinary glucose values of 20, 30 or 15 g in single blood or urine samples. In the latter situation the

physician usually requires further information before being able to decide upon the relative degrees of control of these particular patients.

The advantages of HbA1 quantitation as a means of assessment of metabolic control in diabetic patients can be summarized as follows: (i) it is an objective test and is not dependent on the patient's cooperation or accuracy as is the case with spot checking of urine fractional collections; (ii) it is independent of the time of day, meals and exercise; (iii) it is convenient to be able to describe metabolic control in diabetic patients with a single value; (iv) it facilitates the initial assessment of the patient and simplifies the follow-up process. Specifically, HbA1 is useful in determining the necessity to change from one mode of treatment to another, or to increase or decrease the dose of insulin or oral hypoglycaemic medication which the patient is taking. Also: (v) it provides the physician with a definitive end point towards which therapy should be directed; (vi) it may serve as a screening test for those diabetic patients who appear to be doing well, but are really in less than optimal control.

An additional possible application of HbA1 measurement is for the early diagnosis of diabetes. The quantitation of HbA1 may be a useful adjunct to the glucose tolerance test. It seems likely that $HbA1_c$ will have to be measured specifically in order to increase the method's sensitivity in this circumstance. We are not aware that such a study has been performed.

HbA1 has certain disadvantages as an indicator of metabolic control in diabetes. Thus the HbA1 level is not helpful in deciding upon acute manipulations of insulin therapy or diet. A particular HbA1 level may alert the physician to the need for better control, but would not indicate the time at which an alteration of insulin dosage is required, or the magnitude of the change which is indicated. Obviously HbA1 levels do not reflect the occurrence of hypoglycaemic episodes. In certain haematologic disorders with an abnormal red blood cell life span, HbA1 levels are significantly altered. In infants and pregnant women, HbF may interfere to some degree with the HbA1 assay, limiting its use in these situations. Although the present methodology for measurement of HbA1 is probably too expensive and tedious to be used on a routine basis, commercial laboratories have recently developed a simpler, faster and cheaper assay, which is now being marketed.

Conclusion

The addition of HbA1 to our armamentarium of tests focuses again upon three unsettled, important issues in diabetes:

1. Even though HbA1 may be a suitable indicator of diabetic control, will knowledge of its levels actually lead to an improvement in the metabolic control of the diabetic patient? Do patients, whose physician measures HbA1 concentrations, fare any better than those who do not have this test performed?

2. Is there a limit to our ability to normalize blood sugars of diabetic patients using convential treatment modalities?

3. Are long term diabetic complications prevented or delayed by imposing a rigid regimen aimed at normalising the blood glucose levels?

Although many innovative and meticulously planned clinical research projects will be needed to definitively answer these questions, it seems that we are rapidly developing the technical capability to carry them out. It is in this context that the significance of HbA1 measurements should be viewed.

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Dr. A. H. Rubenstein
Department of Medicine
University of Chicago
950 East 59th Street
Chicago, IL 60637
USA