Originals

HbA₁ in assessment of metabolic control in diabetic BB/E rats

F.J. Tames¹, J.D. Baird² and A.J. Bone³

¹ Department of Clinical Pathology, Manchester Royal Infirmary, Manchester,

² Metabolic Unit, University of Edinburgh Department of Medicine, Western General Hospital, Edinburgh and

³ Professorial Medical Unit, Southampton General Hospital, Southampton, UK

Summary. Estimations of HbA₁ levels have been used to assess long-term glycaemic control in spontaneously diabetic BB/E rats. The degree of metabolic control achieved by once daily insulin injections and continuous insulin infusion by osmotic minipump was compared. Citrate gel electrophoresis of lysed erythrocytes, previously washed and incubated in 0.9% NaCl, gave accurate HbA₁ values without interference from either abnormal Hb variants or labile glycosylation products. Over a 12 week period there was no significant difference in the mean random weekly plasma glucose concentrations between diabetic rats maintained on insulin injections or continuous infusion therapy. The HbA₁ values in the injection-treated animals remained unchanged throughout the study period (mean $\pm$ SEM = $5.1 \pm 0.1\%$). Diabetic rats treated by osmotic minipump showed a steady decline in values over the same period ($4.1 \pm 0.1\%$;

$p < 0.001$ vs injected rats) but levels remained higher than those recorded in non-diabetic control rats ($2.9 \pm 0.01\%$; $p < 0.001$ vs pump-treated rats). These differences in HbA₁ were reflected in the plasma glucose values obtained during a 30 h glucose profile performed after six weeks of insulin therapy. Diabetic rats on injection therapy showed considerable diurnal variation in plasma glucose concentration (5.5 – 11.2 mmol/l; mean 8.9 ± 0.5) but continuous insulin infusion eliminated the fluctuations giving a significantly lower mean glucose level over the 30 h period (7.3 ± 0.1 mmol/l; $p < 0.005$). HbA₁ levels show a poor correlation with random plasma glucose estimations ($r = 0.43$) but provide a simple and accurate assessment of long-term glycaemic control without the need for multiple 24 h glucose profiles.

Key words: HbA₁, BB/E rat, metabolic control

The spontaneously diabetic BB rat displays a diabetic syndrome remarkably similar to human Type 1 (insulin-dependent) diabetes mellitus [1]. In these animals, the apparently rapid onset of the disease is characterised by hyperglycaemia, hypoinsulinaemia and ketosis [2]. These symptoms can be controlled and a degree of glycaemic control maintained by daily administration of insulin allowing the animals a normal lifespan. However, metabolic abnormalities persist even in insulin-treated diabetic rats with apparently normal random glucose levels [3]. Moreover, the diabetic BB rat shows evidence of diabetic microvascular complications including morphological changes in the nervous system [4], retina [5] and kidney [6]. Thus, the BB rat would appear to be an excellent model for investigating the development and reversibility of diabetic microangiopathy in Type 1 diabetes mellitus. Such studies would, however, depend on the availability of accurate methods for measuring, achieving and maintaining various degrees of metabolic control for long time periods.

Measurements of HbA₁ have proved to be a useful and convenient way of monitoring the diabetic control

achieved by different treatment regimens in patients with Type 1 diabetes [7, 8]. HbA₁ levels can be measured by cationic exchange chromatography [9], high pressure liquid chromatography [10] and isoelectric focussing [11]. These techniques are however hard to standardise and apply to studies in different animal models of diabetes where the presence of major Hb variants may make HbA₁ values difficult to interpret [12]. Recently, affinity chromatography has been applied to the measurement of HbA₁ [13] and shown to be a precise method readily applicable to different animal species including rats [14] and mice [15]. This technique does however require two elution steps followed by Hb measurements of the bound and unbound fractions.

The present study describes a rapid method for the measurement of HbA₁ in diabetic rats using the Corning gel electrophoresis system. The method has been used to assess the degree of long-term metabolic control achieved in spontaneously diabetic, insulin-dependent BB/E rats treated either by once daily insulin injection or by continuous subcutaneous insulin infusion (implantable osmotic minipump).

Fig. 1. Electrophoretic distribution profile of Hb components on citrate agar plates (60 V for 40 min at pH 6.3). Bands are stained with 2% amido black 1013 in 5% acetic acid. Lanes 1–4 are samples from diabetic BB/E rats. The corresponding peaks of Hb visualised by automatic scanning densitometry (420 nm) are shown below

Materials and methods

Animals

The animals used in this study were from the Edinburgh colony of BB/E rats, the nucleus of which was kindly donated by Dr. P. Thibert, Animal Resources Division of Canada, Ottawa, Canada in 1982. The colony consists of two lines of animals created by selective breeding. In the high diabetes incidence line, the incidence of insulin-dependent diabetes is 60–70% and the mean age at onset is 96 ± 18 days. In the low diabetes incidence line <2% of animals become diabetic by 120 days of age. Animals were kept on a 12 h light/dark cycle (07.00–19.00 hours) and allowed free access to food and water. Body weight and urinary glucose levels were monitored daily (Diabur test strips; Boehringer Corporation Ltd., Lewes, UK) and onset of diabetes was defined by the first positive urine glucose estimations.

Insulin therapy

Diabetic BB/E rats were maintained normoglycaemic either by: (1) single daily s.c. injections of Ultratard insulin (Novo, Copenhagen, Denmark) (SCI) – the dose being adjusted for individual animals according to daily measurements of body weight and the degree of glycosuria; or (2) the use of Alzet M002 osmotic minipump (Alza Corporation, Palo Alto, Calif., USA) (CSII) for s.c. insulin infusion. Each minipump (an implantable, 6.5 mm $\times$ 2.5 cm, self-contained 2-week infusion device) was filled with Hoe 21 PH U-400 insulin (kindly donated by Dr. M. Zoltobrocki, Hoechst AG, Frankfurt, FRG) and primed to release 6U insulin/24 h for 14 days. The pumps were implanted subcutaneously into the ventral thoracic region of the rat.

Electrophoretic analyses

Mixed venous/arterial blood samples (100 μ l) were collected from the tails of non-anaesthetised animals into heparinised tubes and centrifuged for 2 min at 10,000 rev/min. Plasma was decanted and stored frozen at -20°C for subsequent glucose estimation (Beckman glucose analyser, Beckman Instruments, High Wycombe, UK). The erythrocytes were washed three times in 0.9% (w/v) NaCl and incubated overnight at room temperature with 100 μ l of 0.9% NaCl. The cells were centrifuged and haemolysed by addition of four volumes of deionised water containing 0.1% saponin and 25 μ l toluene. After vortex mixing, the cells were spun for 5 min at 10,000 rev/min and 1 μ l of the haemolysate, containing approximately 40 μ g of rat Hb, was applied to individual sample wells on an electrophoresis plate (Corning Medical and Scientific Ltd., Halstead, UK).

Rat Hb variants were separated by electroendosmosis on citrate agar plates (Corning "Glytrac" electrophoresis system, 1.5 $\times$ 17 cm band width) using 0.1 mol/l citrate buffer (pH 6.3) under 60 V for 40 min. The percentage of HbA₁ was determined by automatic scanning densitometry on a Corning 720 fluorometer/densitometer equipped with a 420 nm filter. After electrophoresis, the different Hb bands could be visualised by staining the gel plate with 2% amido black 1013 in 5% acetic acid.

In vitro experiments

Erythrocytes were obtained from the tails of non-diabetic and SCI diabetic rats and incubated with phosphate buffered saline (0.05 mol/l, pH 7.4) containing 25 mmol/l glucose. Incubation was performed at 37°C in an atmosphere of 95% O₂/5% CO₂ for up to 5 h. Cell samples were divided such that the cells were haemolysed for estimation of HbA₁ either with or without prior overnight incubation with 0.9% NaCl.

Assessment of chronic glycaemic control

Three groups of rats were studied: (1) non-diabetic BB/E rats from the low diabetes incidence line; (2) diabetic BB/E rats treated with insulin by SCI; and (3) diabetic BB/E rats treated by CSII (see above for full details of insulin treatment regimens). There were four rats in each study group. Weekly estimations of plasma glucose and HbA₁

Fig. 2. Changes in HbA₁ concentrations during incubation of erythrocytes in vitro with 25 mmol/l glucose. Symbols indicate erythrocytes from: diabetic BB/E rats with (□) or without (■) overnight incubation in 0.9% NaCl; non-diabetic BB/E rats with (○) or without (●) overnight incubation in 0.9% NaCl

Fig. 3. HbA₁ concentrations and simultaneous plasma glucose values in diabetic BB/E rats. Correlation coefficient between plasma glucose concentrations and HbA₁ levels = 0.43 ($p > 0.05$; $n = 50$)

were performed on all rats from approximately 80 days of age. At onset of diabetes (positive urinary glucose test) rats were randomly allocated to either the SCI or the CSII insulin-treatment groups. Insulin therapy was continued for 12 weeks with replacement of osmotic minipumps, in the CSII group, every 14 days. Glycaemic control throughout the study period was monitored by weekly measurements of plasma glucose and HbA₁ with the blood samples being taken at the same time of day (14.00 hours). In addition after six weeks of study all rats were subjected to a 30 h plasma glucose profile to assess the diurnal variation in glycaemic control. Blood samples were taken for glucose estimations every 2 h from 09.00 to 15.00 hours the following day. The mean amplitude of glycaemic excursions, a measure of diabetic instability [16], was calculated for each group of study animals.

Statistical analysis

Comparison of means was performed by Student's *t*-test for paired sample analysis and regression curves plotted by a least square procedure using a Sinclair "Spectrum" microcomputer (Sinclair Research Ltd., Cambridge, UK).

Results

Figure 1 shows the separation of Hb components by citrate agar electrophoresis in a diabetic BB/E rat. The stained gel shows two main bands: (1) the major component corresponding to human HbA₀ and (2) the faster migrating HbA₁. No abnormal Hb components were found in the rat haemolysates with automatic scanning densitometry showing only two clearly defined peaks.

Reproducibility of the Corning electrophoresis system for measuring HbA₁ in rat erythrocytes was determined using pools of haemolysates stored in liquid nitrogen. This pooled material, obtained from non-diabetic and diabetic BB/E rats, was used as an internal quality control in all subsequent determinations of HbA₁. Blood from a non-diabetic rat was assayed 16 times and blood from a diabetic rat assayed 24 times on three consecutive

Fig. 4. Plasma glucose levels in BB/E rats at onset of diabetes and during a subsequent 12 week period of insulin therapy. Symbols indicate: non-diabetic BB/E rats (●); diabetic BB/E rats maintained by insulin injections (■) or continuous insulin infusion - osmotic minipump (□). Arrows indicate pump replacement

days. Interassay coefficient of variation (CV) for the non-diabetic sample was 7.6% (mean $\pm$ SEM, HbA₁ = $2.8 \pm 0.02\%$) and 6.4% for the diabetic sample (HbA₁ = $8.2 \pm 0.12\%$). The effects of incubating erythrocytes in vitro with 25 mmol/l glucose on HbA₁ values are shown in Figure 2. Values were increased by 54 and 60% in samples for non-diabetic and diabetic rats respectively. Overnight incubation of erythrocytes in 0.9% NaCl completely eliminated the labile component of HbA₁ from both non-diabetic and diabetic samples. Similarly, in vivo in diabetic BB/E rats, the labile component of HbA₁ can increase true HbA₁ values by up to 55% at a mean plasma glucose level of 24.8 mmol/l (results not shown).

Figure 3 shows the HbA₁ values with simultaneous plasma glucose concentrations in diabetic BB/E rats. Random blood samples ($n = 50$) collected (at 14.00 hours) from diabetic rats regardless of treatment or the duration of diabetes showed a poor correlation between plasma glucose concentration and HbA₁ levels ($r = 0.43$; $p > 0.05$).

The effect of long-term insulin treatment, either by s.c. injection or osmotic minipump, on plasma glucose levels in diabetic BB/E rats is shown in Figure 4. Random plasma glucose concentrations in the diabetic animals rose from 5–18 mmol/l during onset of disease but returned to near normal levels within two weeks of commencing insulin therapy. For the remainder of the study period, there was no significant difference in the mean random weekly plasma glucose concentration between the injection-treated, pump-treated or control animals. However, the mean plasma glucose level over the ten week period was significantly higher in the injection-treated rats than in the control rats (6.9 ± 0.6 vs 5.3 ± 0.1 mmol/l; $p < 0.005$).

The HbA₁ levels, determined over the same study period, are shown in Figure 5. The HbA₁ levels in the injection-treated diabetic rats remained essentially unchanged throughout the study period (mean $\pm$ SEM = $5.1 \pm 0.1\%$). However, the diabetic rats treated by osmotic minipump showed a steady decline in values over

Fig. 5. HbA₁ levels in BB/E rats at onset of diabetes and during a subsequent 12 week period of insulin therapy. Symbols indicate: non-diabetic BB/E rats (●); diabetic BB/E rats maintained by insulin injection (■) or continuous insulin infusion – osmotic minipump (□). Arrows indicate pump replacement

Fig. 6. Plasma glucose levels recorded during a 30 h glucose profile performed in diabetic BB/E rats after six weeks of insulin therapy. Symbols indicate: non-diabetic BB/E rats (●); diabetic BB/E rats maintained by insulin injection (■) or continuous insulin infusion – osmotic minipump (□). Arrows indicate insulin injections

the 12 week period and the mean value was significantly lower than that of the rats on injection therapy ($4.1 \pm 0.1\%$; $p < 0.001$). This value was however still higher than that recorded in the control animals ($2.9 \pm 0.01\%$; $p < 0.001$).

Figure 6 shows results from a 30 h glucose profile performed on the study animals after six weeks of insulin therapy. The diabetic rats maintained by insulin injection therapy showed considerable diurnal variation in plasma glucose levels (5.5 – 11.2 mmol/l; mean = 8.9 ± 0.5 mmol/l). Treatment of animals by osmotic minipump eliminated the fluctuations in glucose levels, giving a mean value of 7.3 ± 0.1 mmol/l which was significantly lower ($p < 0.005$) than that of the injection-treated rats but higher than the non-diabetic control animals (mean = 6.1 ± 0.06 mmol/l; $p < 0.001$). To further quantitate differences in glycaemic control, the mean amplitude of glycaemic excursion (MAGE) was calculated by taking the arithmetic mean of the blood glucose increases or decreases over the 30 h period. There was no significant difference in MAGE between non-diabetic control 0.23 (mmol/l) and diabetic rats treated by osmotic minipump (0.44 mmol/l). The in-

jection-treated diabetic rats had significantly higher values of MAGE (2.10 mmol/l) than both pump-treated ($p < 0.01$) and non-diabetic ($p < 0.01$) rats.

Discussion

The use of animal models for the study of the development of Type 1 diabetes and its complications depends on the availability of: (1) an animal displaying a diabetic syndrome similar to human Type 1 diabetes mellitus; (2) a method of accurately assessing glycaemic control, and (3) a means of achieving and maintaining varying degrees of glycaemic control for prolonged periods. In the present study we have described an electrophoretic method for measuring HbA₁ in the spontaneously diabetic BB/E rat. This method has been used to assess the long-term glycaemic control achieved in diabetic animals by different insulin treatment regimens.

Elevated HbA₁ levels have been previously reported in genetically diabetic mice [15, 17] and in both mice [17] and rats [14, 18, 19] with chemically-induced diabetes. The spontaneously diabetic BB/E rat showed increased levels of HbA₁ although the actual values were generally lower than those recorded for streptozotocin-diabetic animals [14, 19]. Differences in methodology could account for this variation but in addition, the present values were determined without interference from either Hb variants or labile fractions. Indeed, similar studies have indicated the importance of removing the labile components before determination of HbA₁ in diabetic patients [20].

Two different methods of insulin treatment were evaluated in the diabetic BB/E rats: (1) conventional s.c. insulin injection (SCI) and (2) continuous s.c. insulin infusion (CSII) via osmotic minipump. Both insulin regimens were effective in lowering the raised plasma glucose values seen at onset of diabetes, to near normal values within two weeks. Indeed, subsequent random plasma glucose estimations, taken at weekly intervals, showed no significant difference between the injected, CSII-treated and/or non-diabetic control animals. These findings were not consistent with the HbA₁ levels observed over the same ten week period, which showed high values in the injection-treated rats but a gradual decline in animals on CSII, albeit not to non-diabetic levels. This inconsistency between plasma glucose and HbA₁ levels was confirmed by the poor correlation between these two parameters observed in 50 random blood samples taken from diabetic BB/E rats (Fig. 3). The present findings contrast with previous reports of a good correlation between plasma glucose and HbA₁ in diabetic rats [14] and mice [15] but are, however, in good agreement with other studies showing a poor correlation in both rats [18] and diabetic patients [21, 22]. Moreover, BB/E rats subsequently developing diabetes had normal plasma glucose levels but elevated HbA₁ values at least two weeks prior to onset of overt hyperglycaemia. Thus, measurements of abnormal Hb glycosylation may be of potential value in the early detection of clinical diabetes.

The studies aimed at achieving and assessing long-term glycaemic control showed that in injected-treated

rats apparent near normal glucose control was not accompanied by any drop in levels. On the other hand, pump-treated animals with similar random plasma glucose concentrations evidenced a gradual decline in values. The reasons for this discrepancy became apparent when plasma glucose levels were monitored in the groups of animals over a 30 h period. Values of MAGE confirmed that there was considerable diurnal variation in glucose concentrations in the rats on injection therapy whilst those on CSII maintained a steady glucose profile albeit with values higher than those recorded in non-diabetic rats. It appears, therefore, that long-term assessment of glycaemic control and diabetic instability in this animal model of human Type 1 diabetes can be achieved by estimations of HbA₁ levels, thus providing an alternative to large numbers of plasma glucose determinations.

In summary, we have shown that it is possible to achieve and maintain different degrees of glycaemic control in the spontaneously diabetic BB/E rat. The metabolic status of the animals can be simply and accurately assessed by the measurement of HbA₁ thus making long-term studies relating metabolic control to the development of diabetic microangiopathy possible.

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Dr. A.J. Bone
Endocrine Section
Medicine II
Level D, South Block
Southampton General Hospital
Southampton SO9 4XY
UK