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The Measurement of Haemoglobin A_{1c} by Isoelectric Focussing in Diabetic Patients

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Summary. Incubation in vitro of human red cells in increasing glucose concentrations results in a rise in both haemoglobin A_{1c} levels and an intermediate band when measured by an isoelectric focussing method. There are strong correlations between blood glucose levels, levels of haemoglobin A_{1c} and the intermediate band both in vitro and in blood samples taken from diabetic patients. As the intermediate band is also included in the measurement of haemoglobin A_{1c} by the usual analytical methods, this may lead to inaccurate results.

Key words: Haemoglobin A_{1c}, intermediate band, isoelectric focussing, diabetic control, blood glucose.

There are several methods available for the measurement of haemoglobin A_{1c} (HbA_{1c}) but few can measure HbA_{1c} distinctly from the intermediate Schiff base. As the kinetics of formation and reversal of the Schiff base are likely to be different to that of the formation of HbA_{1c}, it is likely that any significant concentration of the Schiff base will cause a significant over-estimation in the measurement of HbA_{1c}. However, using an isoelectric focussing method, it is possible to differentiate between HbA_{1c} and this intermediate band [1–2]. This paper reports the results of using this method in investigations in vivo and in vitro of the relationship between HbA_{1c}, the intermediate band and blood glucose levels, and particularly whether short term fluctuations in blood glucose levels can influence significantly HbA_{1c} levels as reported by Svendsen [3].

Patients and Methods

Methods

Whole blood (1 ml) was washed with 0.15 mol/l NaCl; the washed red cells were then haemolysed in 0.01 mol/l potassium cyanide and four drops of carbon tetrachloride added. The haemolysate was separated from the red cell debris by centrifugation. 15 µl of the haemolysate was applied to the cathode end of a pre-focussed polyacrylamide gel plate incorporating a gel spacer (β-alanine, pI 6.9) to facilitate HbA and HbA_{1c} separation (LKB Instruments, Bromma, Sweden). The sample was then electro focussed at 20 W at a constant 15 °C for approximately 1.5 h. When electro focussing was complete (Fig. 1), the discrete HbA_{1c} band and the band imme-

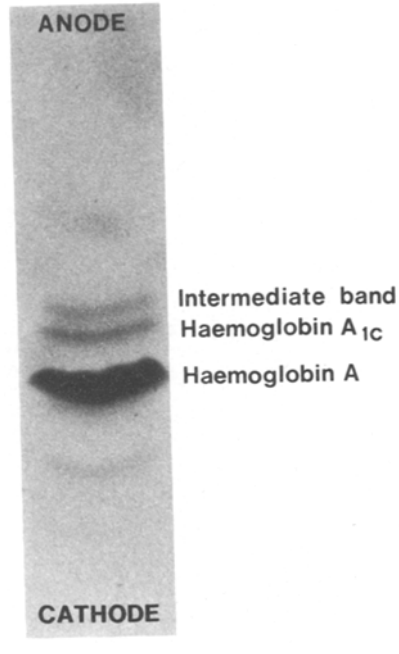

Fig. 1. Isoelectric focussing plate showing HbA, HbA_{1c} and the intermediate band

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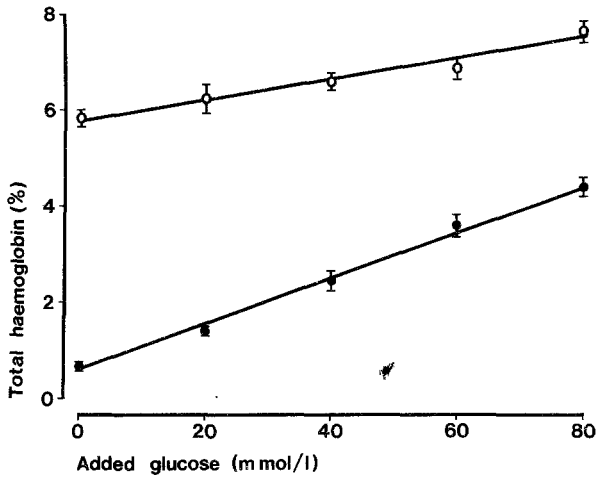

Fig. 2. The relationship between HbA_{1c} (○—○) and intermediate band (●—●) levels after incubation of red cells in increasing glucose concentrations for 2 h at 37 °C. Results expressed as mean ± SEM

Table 1. Correlation coefficients between blood glucose, HbA_{1c} and intermediate band levels in incubation study in vitro and in 57 diabetic patients

Variables	In vitro ($n = 6$)	In vivo ($n = 57$)
Blood glucose versus HbA_{1c} levels	$r = 0.84$ $p < 0.001$	$r = 0.70$ $p < 0.001$
Blood glucose versus intermediate band	$r = 0.96$ $p < 0.001$	$r = 0.75$ $p < 0.001$
Intermediate band versus HbA_{1c} levels	$r = 0.79$ $p < 0.001$	$r = 0.62$ $p < 0.001$

Table 2. Levels of blood glucose and HbA_{1c} in 303 diabetic patients on various anti-diabetic therapies and the correlation between them in each group

	Diabetic patients on:		
	Diet alone	Hypoglycaemic drug therapy	Insulin therapy
No. of patients	22	111	170
Blood glucose (mmol/l)	7.20 ± 0.49	9.30 ± 0.33	10.50 ± 0.34
HbA_{1c} level (%)	9.93 ± 0.18	9.04 ± 0.46	10.95 ± 0.15
Correlation coefficient: blood glucose versus HbA_{1c}	0.21	0.72	0.44
p	NS	< 0.001	< 0.001

Results are expressed as mean ± SEM

diately anodic to it (intermediate band) were cut from the plate using a fine scalpel. The bands were eluted in a Tris/HCl buffer (pH 8.3) containing 0.1 mmol/l EDTA, overnight at 4 °C with end-over-end mixing. The concentrations of HbA_{1c} and the intermediate band were expressed as a percentage of the total haemoglobin of the haemolysate measured by spectrophotometry at 415 nm (Pye Unicam SP 1800). Blood glucose was measured by a glucose oxidase method (Yellow Springs Instrument Company).

For the studies in vitro, 10 ml blood were taken from six non-diabetic volunteers (mean age: 30 years; mean blood glucose: 3.6 mmol/l) and 1.0 ml samples of whole blood were incubated at 37 °C for 2 h in 0.15 mol/l NaCl containing increasing glucose concentrations (10–80 mmol/l). The red cells were then haemolysed and HbA_{1c} and intermediate band measured in duplicate.

Patients

To investigate the relationship between blood glucose, HbA_{1c} and intermediate band levels in vivo, postprandial blood samples were taken from 57 patients who were attending the diabetic clinic. In 303 patients who attended the diabetic clinic regularly HbA_{1c} levels were also measured by isoelectric focussing. Fifty-six percent of patients were receiving insulin therapy, 37% oral drug therapy and 7% diet alone.

All results are expressed as mean ± SEM and correlation coefficients. Linear correlations were calculated by a standard method.

Results

The intra-assay coefficient of variation for HbA_{1c} measurement was 2.8% and for the intermediate band was 6.4%. Normal non-diabetic HbA_{1c} levels were 5.85 ± 0.16% and intermediate bands 0.68 ± 0.06% at a mean blood glucose level of 3.6 mmol/l.

The effects of increasing the concentration of glucose in isotonic saline on the production of HbA_{1c} and the intermediate band during 2 h incubations are shown in Figure 2. A linear increase occurred in both products over the range of glucose concentrations studied, although the slopes of the lines were different. A fivefold increase in the intermediate band was detected when whole blood was incubated in 80 mmol/l glucose; a smaller increase (31%) occurred in HbA_{1c} at this glucose concentration. Under these conditions there are significant correlations between the levels of glucose and HbA_{1c} and the intermediate band (Table 1). The same strong correlations also exist between these factors in the measurements in vivo in 57 diabetic patients (Table 1). In these patients the mean blood glucose levels were 10.9 ± 0.32 mmol/l, HbA_{1c} 11.94 ± 0.64% and intermediate band 1.53 ± 0.08%.

Table 2 shows the mean blood glucose and HbA_{1c} levels in each of the diabetic treatment groups and their correlation coefficients. Only a weak correlation was found between blood glucose and HbA_{1c} levels in those patients on diet alone, but there was a strong

correlation in those patients on oral hypoglycaemic therapy with that in patients on insulin less strong.

Discussion

There has been debate as to whether haemoglobin A could undergo rapid glycosylation to HbA_{1c} when the blood glucose level rises in the short term [3, 4]. These results in vitro suggest that a small but definite rise in HbA_{1c} does occur but with a much more marked elevation in the intermediate band. This latter band would be measured in other methods, particularly in the micro-column method [5] which would explain the different results obtained by other workers. However the change in HbA_{1c} levels is not great over those blood glucose level changes which might be expected to occur in clinical diabetes, i.e. up to 30–40 mmol/l, whereas the increase in the intermediate band is three- to fourfold at these concentrations. A significant rise in the intermediate band after the incubation of red cells for 2 h at 37 °C in 100 mmol/l glucose concentration has been reported by Mortensen [1] using isoelectric focussing although he failed to detect any increase in HbA_{1c} levels.

Our findings of a strong positive correlation between HbA_{1c} and blood glucose levels in a group of diabetic patients controlled on oral hypoglycaemic agents was not unexpected as random blood glucose levels in this group of patients are more representative of mean blood glucose over the previous weeks, in contrast to insulin requiring diabetics who have much greater variation of blood glucose levels.

We do not feel that our results negate the usefulness of HbA_{1c} determinations as an index of diabetic control. However a cautionary note should be sounded; unless the method used for the determination of glycosylated haemoglobin can accurately separate HbA_{1c} from its intermediate condensation product, or precautions are taken to remove the intermediate product [2], considerable overestimation of HbA_{1c} may occur. At present we would suggest that isoelectric focussing represents the best method for the direct measurement of HbA_{1c}.

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