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An enzyme immunoassay for advanced glycosylation end-products in serum

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Abstract

We successfully developed an in-house, competitive enzyme immunoassay to measure advanced glycosylation end-products (AGE) in serum. The assay involved coating microtitre wells with AGE-BSA at 8 µg/ml for 4 hours, followed by overnight incubation of 20 @ sample (prediluted at 1:6) with 80 µl antiserum (1:8000). HRP-labelled goat anti-rabbit was used as the second antibody and 3,5',5, 5'-tetramethylbenzidine dihydrochloride as the substrate. Incubation was carried out at 4°C. As suggested in an earlier study, we standardised the AGE units against normal human serum (NHS). Thus, one AGE unit was defined as the inhibition that resulted when the 1:6 diluted NHS was assayed. Mean (± SD) AGE level in normal subjects (n = 37) was significantly lower than in diabetes subjects with microalbuminuria (n = 57) (6.0 ± 0.7 versus 10.2 ± 4.7 units/ml, p = 0.0001). With the availability of in-house assay and by standardising the AGE unit with the other laboratories, more studies could be undertaken and results compared, and possibly, further elucidate the roles of AGE in the pathogenesis of diabetic complications.

Key words: AGE, advanced glycosylation end-products; EIA, enzyme immunoassay;

INTRODUCTION

Glucose reacts nonenzymatically with proteins to form chemically reversible early glycosylation products, the Schiff bases, which with time, rearrange to form the Amadori products.' In patients with diabetes mellitus, the amount of these products increases when the blood glucose levels are high but returns to normal when the glucose levels are brought to normal. However, in persistently hyperglycemic state, as in chronic diabetes, Amadori products would undergo chemical rearrangements to form the irreversibly bound, advanced glycosylation end-products (AGE).' As such, AGE accumulate continuously over the years, causing pathological changes and damage to long-lived structural proteins such as the lens protein, nerve myelin and vascular collagen. AGE have been implicated to play major roles in the development of diabetic complications.[3-5]

To date, there is no commercial method available for quantifying AGE. As AGE may serve as a useful biochemical marker of diabetic complications, and perhaps useful when monitoring disease progression or therapy, we therefore undertake this study, to develop an in-house enzyme irnmunoassay (EIA) specific for the measurement of AGE in serum samples.

MATERIALS AND METHODS

The methodology described in this study, including the various procedures to develop and optimise the assay conditions were similar to that reported by Nakayama *et al.*[6,7,8] with some modifications.

Preparation of buffers

All chemicals used in this study were of Analar grade, purchased from Sigma Chem. Co., St Louis, USA. The amount of chemicals quoted below is for preparation of one litre solution.

Phosphate buffered saline (PBS, pH 7.2)
Na_2PO_4 1.28g; KH_2PO_4 0.13 g; NaCl 9.0 g; thiomersal 0.1 g.

Coating buffer (pH 9.6)
Na_2CO_3 1.5g; $NaHCO_3$ 2.94g. Stable up to 2 months at 4°C.

Blocking buffer
PBS containing 2% (weight/volume) BSA. Prepared when required.

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Citrate-phosphate buffer
One capsule of phosphate-citrate buffer with sodium perborate (Sigma, P-4922) dissolved in 100 ml deionized water. Stable up to 1 month at 4°C.

Substrate buffer
One tablet of 1 mg 3,5',5,5'-tetramethylbenzidine dihydrochloride (Sigma T-3405) dissolved in 10 ml of citrate-phosphate buffer. Prepared within 30 minutes of use.

Wash *buffer*
PBS containing 0.05% Tween.

Preparation of immunogen (AGE-KLH) and production of antiserum

Briefly, AGE-KLH was prepared by incubating 100 mg of keyhole limpet-hemocyanin (KLH) with 3 g glucose in 5 ml sterile PBS for 12 weeks at 37°C. The solution was extensively dialysed with PBS before being used as the immunogen. The antibody to AGE-KLH was raised in New Zealand white rabbits. Each rabbit was given intradermal, multiple sites injection with 100 µg AGE-KLH emulsified in equal volume of Freund's Complete Adjuvant (FCA), then boosted with similar preparation at day 14 and day 28. Thereafter, the immunogen was prepared in incomplete FCA and rabbits were boosted every 2 weekly. Test bleed was carried out after 60 days, and additional booster was continued at 4 weekly interval until a satisfactory titer was obtained.

Preparation of AGE-proteins

The AGE-bovine serum albumin (AGE-BSA), AGE-human serum albumin (AGE-HSA) and AGE-bovine pancreatic ribonuclease A (AGE-Rnase A) were prepared by incubating 1 g of each protein with 3 g of glucose in 5 ml sterile PBS. The solution was kept at 37°C for 28 days, extensively dialysed in deionised water, and kept frozen at -60°C until used. A similar procedure was also used to prepare the early forms of glycated-BSA; incubation period with glucose solution was for 7 and 14 days. Reduced AGE-BSA was prepared by incubating 2 mg of the AGE-BSA in PBS containing 100 mM sodium borohydride for 30 minutes at 37°C. Dialysis was carried out for 48 hours at 4°C, with several changes of PBS.

Enzyme immunoassay (EZA) for age

Nunc Immunoplate Maxisorp F96 (with certificate, Nunc, Roskilde, Denmark) 96-well flat-bottomed microtitre plate was used. In this competitive EIA, samples were assayed in triplicate, all incubations were at 4°C, and the solution was kept mixed during incubation using a rotator. The assay procedure is summarised in Fig 1. Each well was first coated with 100 µl of AGE-BSA (8 µg per ml of coating buffer) and after 4 hours, washed 3 times with washing buffer, tapped dry and then refilled with 150 µl of blocking buffer for another hour. After discarding the blocking buffer and drying the plate on absorbant paper, each well was then filled with 80 µl of anti-AGE-KLH (1:8000) and 20 µl of sample which had been prediluted at 1:6 with PBS. The plate was incubated for 18-20 hours, washed, then filled with 100 µl/well of HRP-labelled goat anti-rabbit (1:3000). After 2 hours' incubation and 3 cycle washings, 100 µl/well of substrate solution was added. Colour reaction was allowed to develop in the dark for about 20-30 minutes, and the reaction stopped with 50 µl 1.25M sulphuric acid. Absorbance was read at 450 nm, with reference at 620 nm. The assay performance was monitored using a set of in-house quality control sera consisting of 3 different levels of AGE.

RESULTS

Assay conditions such as the antibody dilution, amount of AGE-BSA used for coating, sample dilution, temperature and time of incubation were investigated and optimised in order to obtain a sensitive assay for detecting AGE in serum. Results were expressed as B/Bo, calculated as:

$$\frac{\text{Experimental OD - Background OD (no antibody)}}{\text{Total OD (no competitor) - Background OD.}}$$

Based on the dose-dependent competition curves, the best displacement and maximum binding was obtained with the AGE-BSA prepared after 28 days (Fig. 2). The glycated-BSA obtained at incubation period of 7 and 14 days with glucose solution were less imrnunoreactive. Thus, the AGE-BSA produced after incubating BSA with glucose solution for 28 days was selected as the coating antigen. Optimal displacement of AGE-BSA at concentrations between 6.25 and 100 µl/ml was obtained when the final dilution of the antiserum was at 1:10,000 and the coating antigen concentration was at 8 µl/ml (Fig 3). There was no significant change in the displacement pattern of the standard curve when the AGE-BSA standards were replaced with its reduced form (Fig. 3). The antiserum was also found to exhibit

Pipette 100 µl diluted **AGE-BSA** into each well

↓ Incubate for 4 h

Wash & dry. Pipette 150 µl 5% BSA

↓ **Incubate** for 1 h

Pipette 20 µl **samples/standards** + 80 µl **anti-AGE-KLH**

↓ Incubate for 18-20 h

Wash & dry. Pipette 100 µl **HRP-GAR**

↓ Incubate for 2 h

Wash & dry. Pipette 100 µl substrate buffer

↓ Incubate for 15-30 min

Pipette 50 µl 1.25M **sulphuric** acid

↓

Measure absorbance at 450 nm, reference 620 nm

FIG. 1: Flow-chart for AGE EIA.

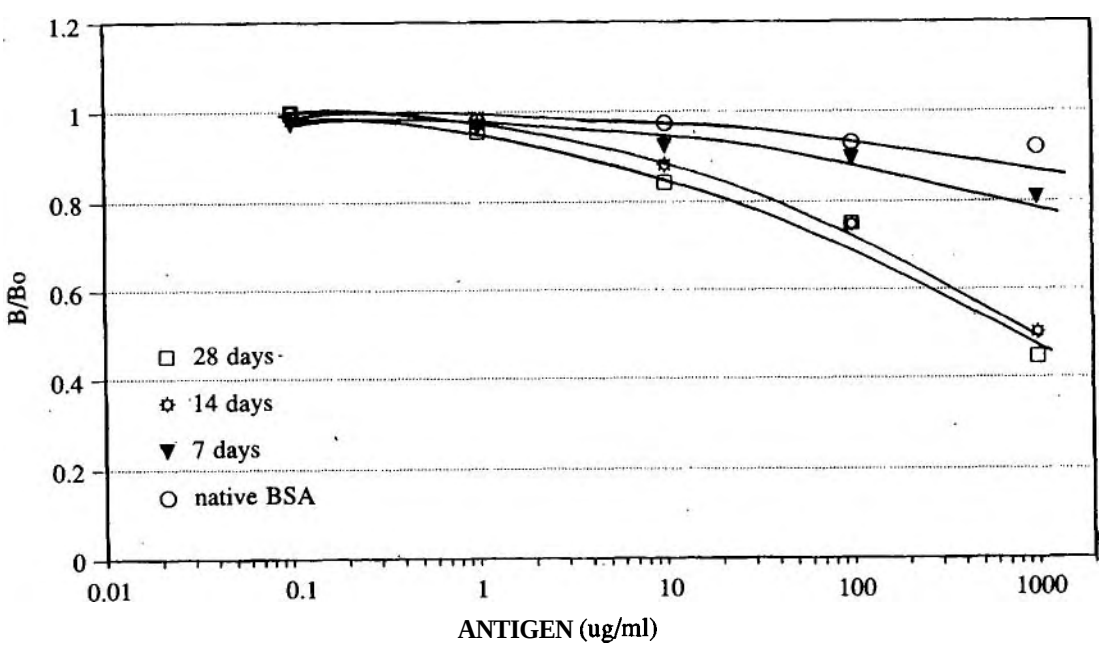

FIG. 2: Time-related increase in immunoreactivity of **BSA** incubated with glucose.

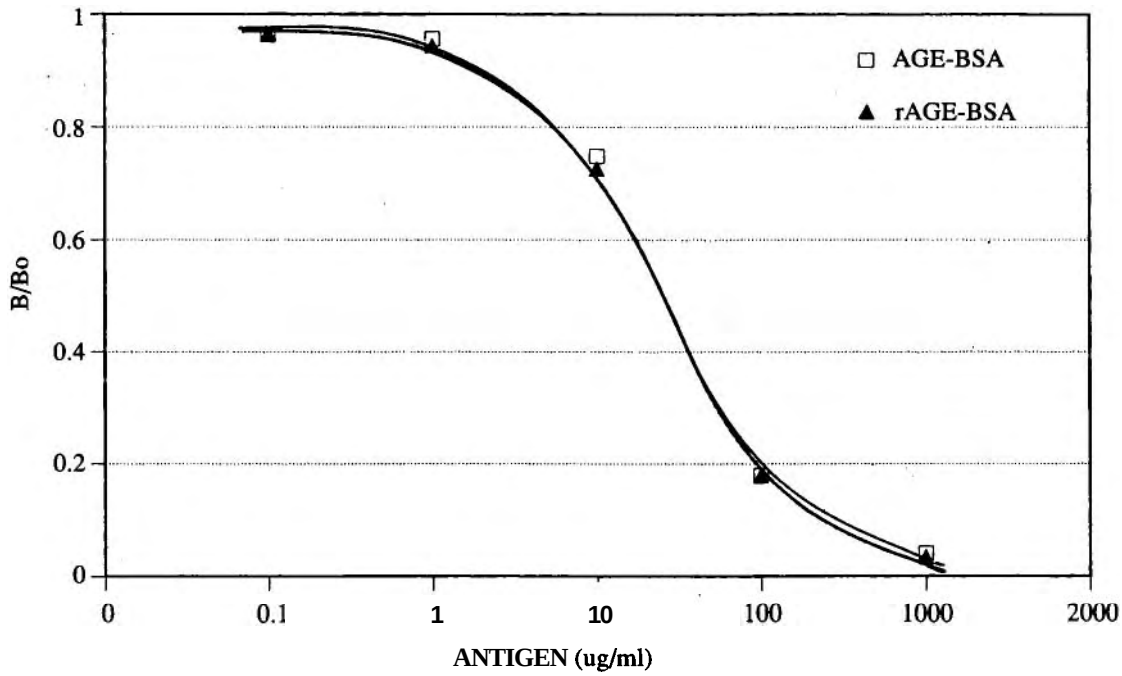

FIG. 3: Competitive EIA inhibition curves for AGE-BSA and chemically reduced AGE-BSA.

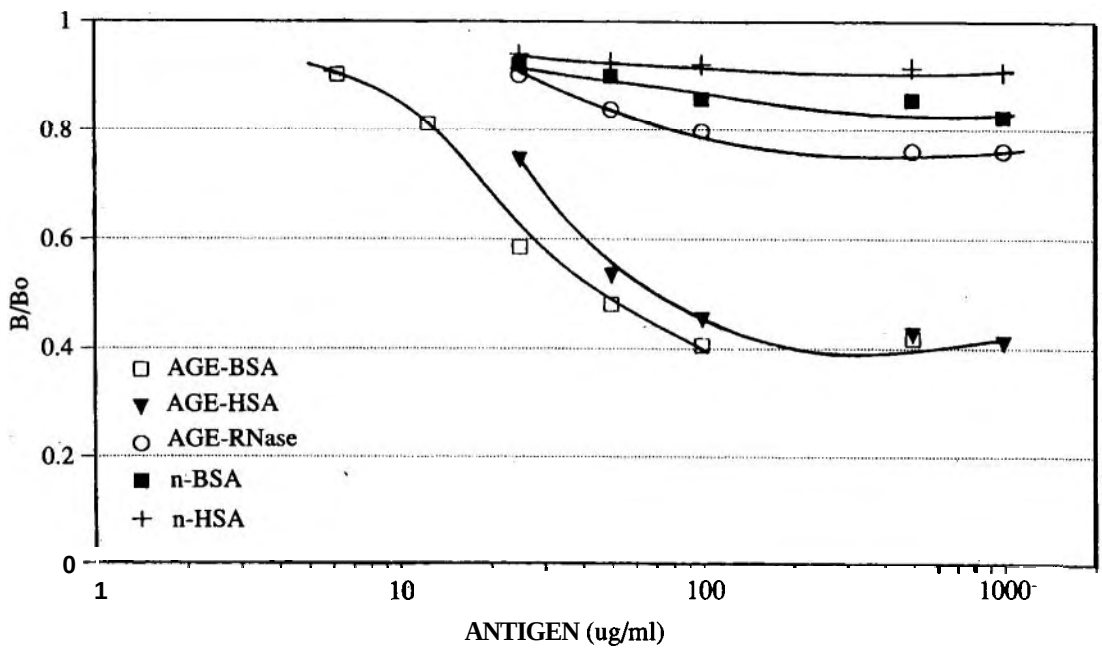

FIG. 4: Immunoreactivity curves of various AGE-proteins and related compounds with anti-AGE-KLH.

high affinity to AGE-HSA and AGE-Rnase but not to the native proteins (Fig. 4).

Optimum detection of AGE in serum samples were achieved when samples were assayed prediluted at 1:6 in PBS. We have also adopted similar procedure proposed by Mitsuhashi et *al.*[9] to define the AGE unit. Thus, one AGE unit was defined as the inhibition that resulted when normal human serum (NHS) was assayed at the 1:6 dilution. We analysed 37 serum samples collected from our normal subjects, aged between 7-30 years and for comparison, 57 serum samples from a group of diabetic patients with microalbuminuria. Percent inhibition in a sample was calculated as [(1 - B/Bo) x 100%]. The mean percent inhibition of the NHS was found to be 24.4 ± 3.1, and by definition, was thus taken to be equivalent to one AGE unit. Since the samples were assayed at a dilution of 1:6, the mean AGE level in the normal subjects was thus 6 unitslml (6.0 ± 0.7 unitslml, mean ± SD), significantly lower than the mean level of 10.2 ± 4.7 unitslml measured in the diabetes patients with microalbuminuria (p=0.0001, Fig. 5). The intra-assay (n = 6) coefficient of variations (CVs) at AGE levels of 8.0, 27.0 and 56.0 unitslml were 11.9, 7.9 and 7.6% respectively while the corresponding inter-assay CVs (n = 11) were 15.1, 11.1 and 9.6% respectively.

DISCUSSION

In vivo nonenzymatic glycosylation of proteins is a continuous process, rapidly reaching equilibrium level at a rate proportional to the glucose concentration in the circulation.' These early glycosylation products, labile Schiff base adducts and Amadori rearrangement products, are not stable and could be chemically reduced to glucitol-lysine by reducing agents such as sodium borohydride[10] In contrast, AGE, formed over long periods of time, are chemically stable compounds. A number of these heterogenous group of irreversibly-modified adducts have been structurally identified, including pentosidines.[11] pyrraline[12] and 2-furoyl-4(5)-(2-furany)-1H-imidazole.[13] It is therefore important that the antibody used in the immunoassay recognised only AGE and not the early stage compounds, and similarly, that the coating antigen used in the competitive assay be of the stable form. As shown in this study, the AGE-BSA obtained after 28 days' incubation with high concentration of glucose was chemically stable. Even after being chemically reduced with sodium borohydride, the standard curve was similar and parallel to that produced by the non-reduced form (AGE-BSA), suggesting that there was no structural change in the antibody-binding sites.

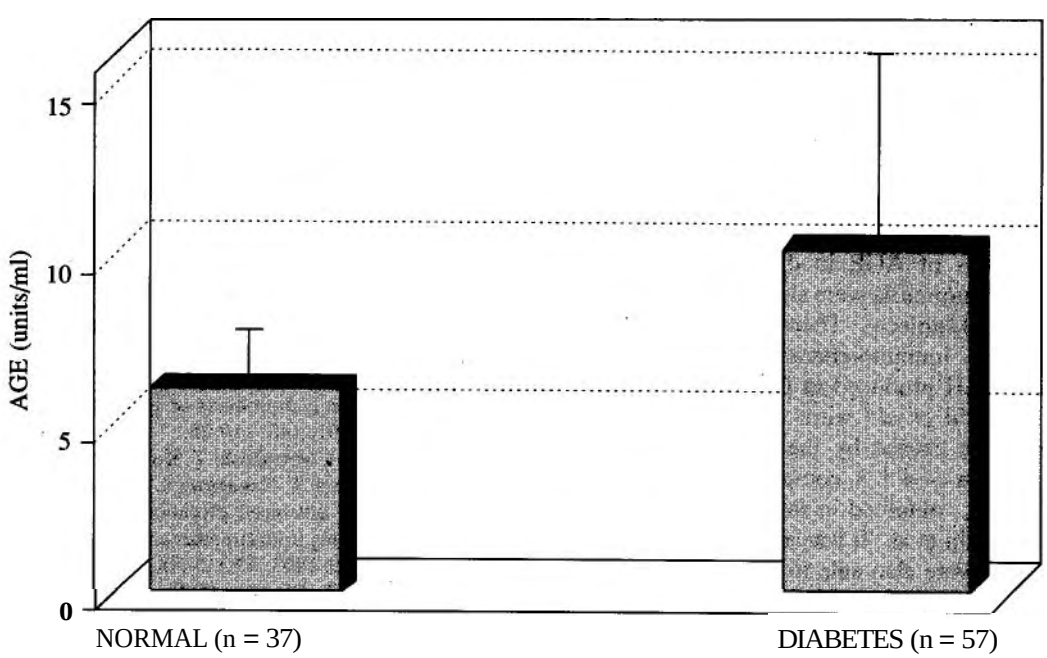

FIG. 5: Mean (± SD) serum AGE level in normal subjects and diabetes subjects with rnicroalbuminuria (6.0 ± 0.7 versus 10.2 ± 4.7 units/ml, p = 0.0001).

Comparable to the observations by Nakayama *et al*,[6] we have also found that there was a time-related increase in immunoreactivity in the BSA incubated with glucose, where highest binding was obtained with the BSA incubated for the longest period (28 days). The cross-reactivity study has indicated that the antiserum produced in our laboratory has very high affinity towards AGE-HSA, which we feel, is one of the important AGE-proteins that play a role in development of diabetic complications.

There are several methods being used to measure AGE. Although fluorescence was often used, the technique has been shown to be less specific.[14,15] Of late, enzyme immunoassay has been the method of choice to quantitate AGE in tissue and serum.[6,9,16] However, being in-house assays and without the availability of an absolute AGE standard, there is no standardisation, making inter-laboratory comparison of results impossible. In the method used by Nakayama *et al*.,[6] one AGE unit was arbitrarily defined as the amount needed to cause 50% inhibition while in the method by Makita *et al*,[16] one AGE unit was defined to be equal to the B/Bo of 1 µg AGE-BSA used as the standard. Further, the quality of AGE-protein used as reference standard and in the antibody production can vary not only between preparations but also on the types of reducing sugars and protein-carrier used.[9] In an effort to overcome these problems, we had therefore adopted the method used by Mitsuhashi *et al*.[9] in defining the AGE unit. We do agree that this method has its limitations; the AGE unit is just a relative measurement against normal human serum and is very dependent on the immunoreactivities of the antibodies. However, consistent with the observations made by other researchers? [16] we have also found that the levels of AGE in diabetes subjects with microalbuminuria were significantly higher than in normal subjects. There is also the possibility that the immuno-characteristics of the anti-AGE-KLH produced in this study and that by Mitsuhashi *et al*.[9] were similar. The percent inhibition caused by the diluted NHS in this study was 24.4 ± 3, comparable to the value of 24.2 ± 5, obtained in the assay developed by Mitsuhashi et al. It was also interesting to note that we were also able to achieve similar assay sensitivity; the optimal dilution for serum samples was 1:6 in our in-house assay, as compared to 1:5 in their enzyme-linked immunosorbent assay.

In summary, given the critical roles that AGE play in the pathogenesis of diabetic complications, there is indeed a need to standardise its measurement. By standardising the method and AGE unit, comparison of results between laboratories would thus be possible and thereby providing more meaningful information and interpretation of results especially if AGE is to be used as the early marker for diabetic complications.

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