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Determination of anti-AGE antibodies in human serum

STEFFAN BAYDANOFF[1], EMILIANA KONOVA[1]* and NATALIA IVANOVA[2]

[1]*Departments of Biology/Immunology and*
[2]*Neurology, University School of Medicine, Pleven, Bulgaria*

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The aim of this study was to develop an immunoenzyme method for the determination of anti-AGE antibodies in human serum. Human aortic elastin glycated *in vitro* (AGE-elastin) was used as an antigen, expressing AGE-epitopes, common to all glycated proteins.

Polyclonal serum from guinea-pig against AGE-Hemocyanin was obtained according to Nakayama *et al.* [(1989) *Biochem Biophys Res Commun* **162**: 740–45] and its specificity was tested via direct and competitive ELISA. Sera of 20 type 1 diabetic patients and 20 healthy subjects were tested using the method described. Seventeen patients had elevated levels of competing factors that may be anti-AGE antibodies, compared with the healthy group. The method could be used for investigation of different clinical groups of type 1 diabetic patients. Such a study would help in understanding the pathogenic role of autoantibodies against advanced glycation end products of proteins for the development of long-term diabetic complications.

Keywords: anti-AGE antibodies, human serum, diabetes

Introduction

The increased non-enzymatic glycation of proteins is a consequence of hyperglycaemia in diabetes and correlates with the severity of diabetic complications [1, 2]. Non-enzymatic attachment of glucose to ϵ-amino groups of lysine and terminal aminoacids leads to formation of Schiff-bases and Amadori-rearrangement products. Amadori products can give rise in time to a family of highly cross-linked, irreversible compounds, designated advanced glycation end products (AGEs), derived from the rearrangement of the initial adducts. The tissue accumulation of AGEs alters the structure and function of long-lived proteins. AGEs could contribute to tissue damage by two pathways: directly and indirectly via cell-mediated interactions. The direct damage is due to cross-linking, polymerization, decreased solubility and sensitivity to enzyme degradation of connective tissue [3, 4], trapping of extravasated plasma proteins [5–7] or inactivation of NO, an important component of endothelium relaxing factor (EDRF) [8]. Cell-mediated interactions are performed via AGE-specific receptors which have been established on macrophages [9], lymphocytes [10], endothelial cells [11], fibroblasts [12], smooth-muscle cells [13] and mesangial cells [14].

The pathogenic role of AGE as an immunogen is not completely clear. It has been established that AGEs have an antigenic similarity, regardless of the protein, on which they have been formed [15]. To date, anti-AGE antibodies and the effect of the duration and severity of diabetic complications on their formation have not been investigated. The aim of this study was to develop an immunoenzyme method for determination of antibodies against advanced glycation end products in human sera, using human aortic AGE-elastin.

Material and methods

Human insoluble elastin was prepared from macro- and microscopic unaltered regions of thoracic aortas, obtained from 10 healthy accident victims, 18–30 years old, using the method of Starcher and Galione [16]. Aminoacid analysis of the purified elastin showed quantitative similarity to the elastin, previously purified [16], and the lack of methionine suggested a low level of contamination. Soluble α-elastin was obtained by the method of Partridge *et al.* [17].

*To whom correspondence should be addressed.

AGE-elastin was obtained via incubation of α-elastin (1.33 mg ml^{-1}) with 100 mmol l^{-1} glucose, in 0.2 M phosphate buffer, pH 7.8, containing 0.04% sodium azide, at 37 °C for 30 days. The fluorescence of the glycated elastin was measured as an index of advanced glycation [18].

Polyclonal immune serum against advanced glycation end products of Hemocyanin from Keyhole Limpets (AGE-KLH) was produced in a guinea pig according to Nakayama *et al.* [15]. KLH (Sigma) – 20 mg ml^{-1} was glycated *in vitro* with 3.33 M glucose in 0.4 M phosphate buffer, pH 7.5 with preservative 0.04% sodium azide, at 37 °C, for 12 weeks. The formation of AGE-KLH was determined via the fluorescence method: 370 nm excitation/440 nm emission. The specificity of the immune serum was tested via direct ELISA for the determination of the titre of anti-AGE-KLH anti-bodies. Briefly, a 96-well microtitre plate was coated with AGE-KLH by adding 100 μl of a solution of AGE-KLH (5 μg ml^{-1}, dissolved in 0.05 M carbonate buffer, pH 9.6) to each well and incubating for 2 h at 37 °C and overnight at 4 °C. Wells were washed with a solution of PBS, containing 0.05% Tween 20 (PBS-Tween) and then blocked by incubation for 1 h with 1% calf serum albumin (CSA) in PBS-Tween. After washing with PBS-Tween, 100 μl of immune serum samples, diluted from 1:500 to 1:128 000 in PBS-Tween was added. The plate was incubated for 1 h at 37 °C. Wells were then washed with PBS Tween, incubated with a peroxidase-linked anti-guinea pig IgG (diluted 1:300 000 in 1% human serum albumin) and reacted with *o*-phenylenediamine as colorimetric substrate after washing. The reaction was terminated by 4 M H_2SO_4 and the absorbance was read at 492 nm was read on a semi-automatic micro-ELISA plate reader.

The specificity of anti-AGE-KLH immune serum was tested further by direct ELISA, using the homologous and different glycated and non-glycated heterologous proteins as coating antigens - α-elastin, AGE-elastin, bovine serum albumin (BSA), glycated bovine serum albumin (AGE-BSA), and collagen type 1 (Sigma). Furthermore, the specificity of the immune serum was tested via competitive ELISA, using the same antigens as experimental inhibitors. Samples of the immune serum, diluted 1:1000 in PBS, were preincubated with 200 μg ml^{-1} solutions of each inhibitor for 2 h at 37 °C and overnight at 4 °C. The reactivity of such treated serum samples with the homologous and heterologous antigens was tested and compared to the reactivity of untreated serum, which was assumed to be 100%.

An immunoenzyme method was developed for the determination of anti-AGE antibodies in human serum on the basis of a blocking ELISA. The wells of the polystyrene plate were coated with AGE-elastin by adding 100 μl of a solution of AGE-elastin (5 μg ml^{-1}, dissolved in carbonate buffer, pH 9.6). After washing with PBS-Tween the wells were incubated with 100 μl from the tested human sera (diluted 1:20 in 1% CSA PBS) for 1 h, at 37 °C. The plate was washed and the wells were incubated with 100 μl anti-AGE-KLH immune serum (diluted 1:500 in 1% CSA PBS-Tween) for 1 h, at 37 °C. The plate was then washed and peroxidase-linked anti-guinea pig IgG, diluted 1:30 000 in 1% human serum albumin was used for 1 h incubation at 37 °C. After incubation with the substrate solution (0.8 mg *o*-phenylenediamine in 0.05 M citrate buffer, pH 5.0 + 0.01% H_2O_2) for 1 h, the reaction was terminated with 50 μl 4 M H_2SO_4. The reactivity of the immune serum without human serum was used as a control.

Anti-AGE antibodies in sera of 20 patients with type 1 diabetes mellitus and 20 healthy aged-controls were determined using the method described. The patients were selected on the basis of the following criteria: age – from 32 to 40 years; duration of diabetes – from 8 to 12 years; current hypoglycaemic treatment – diet control and insulin, lack of hypertension and current illness (including surgery in the past 2 years); normal renal function defined as a serum creatinine concentration of less than 134 μmol l^{-1} and absence of proteinuria detected by Multistix (AMES, England); serum cholesterol and triglycerides in normal ranges; diabetic eye complication – non-proliferative retinopathy with duration from 6 months to 2 years.

Results and discussion

Results obtained by fluorescence estimation of AGE-KLH are shown in Fig. 1. The plateau of fluorescence of the sample was reached 11 weeks after beginning glycation, confirming the formation of AGEs.

Results from investigation of the binding of anti-AGE-

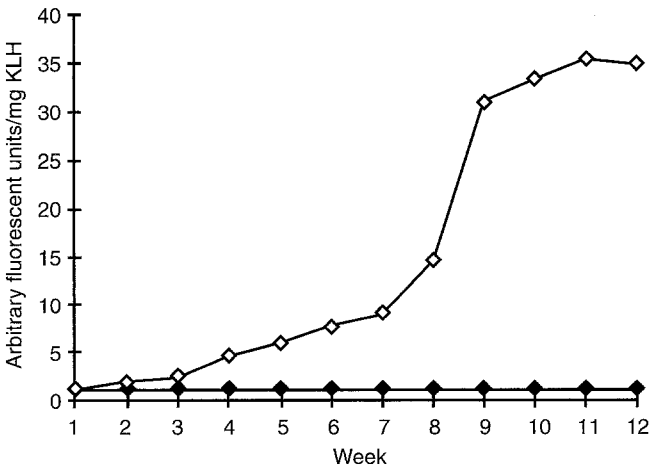

Figure 1. Fluorescence of KLH incubated with 3.33 M^{-1} glucose and diluted to 1 mg ml^{-1} (◇), control–KLH–1 mg ml^{-1} (◆).

KLH immune serum to homologous and different heterologous antigens are shown in Figs 2 and 3. The immune serum had a high titre of anti-AGE-KLH antibodies, lower titres of anti-KLH, anti-AGE-elastin and anti-AGE-BSA antibodies, and low titres of anti-elastin, anti-BSA and anti-collagen Type 1 antibodies. These findings gave us reason to assume that 1:500 dilution of the serum eliminates cross-reactive antibodies when AGE-elastin was used as coating antigen and the binding is due to the specific anti-AGE antibodies. Binding of the immune serum, diluted 1:500 with AGE-elastin and the lack of reactivity with α-elastin, could be explained by antigen differences between both elastins after glycation. Most probably new AGE-epitopes have been formed, recognized by anti-AGE antibodies.

The specificity of the immune serum was tested by competitive ELISA after preincubation of the immune serum diluted 1:1 000 with the homologous and hetero-

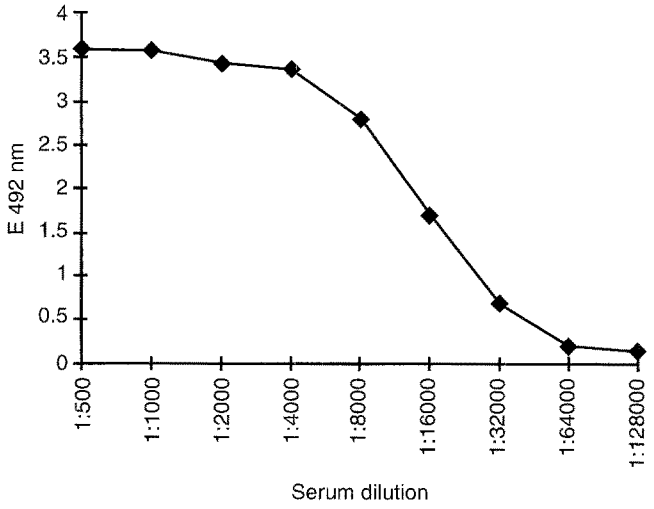

Figure 2. Titre of anti-AGE-KLH antibodies in the guinea pig immune serum against AGE-KLH.

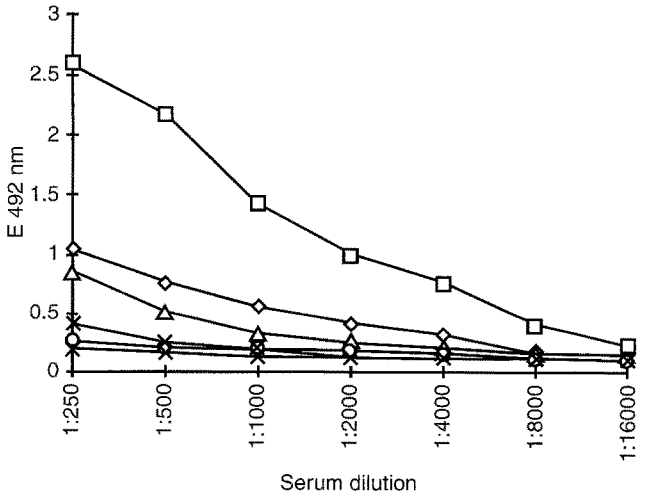

Figure 3. Results from direct ELISA for determination of the reactivity of anti-AGE-KLH immune serum with heterologous coating antigens - AGE-elastin (◇), α-elastin (×), AGE-BSA (△), BSA (○), Collagen Type I (×), KLH (□).

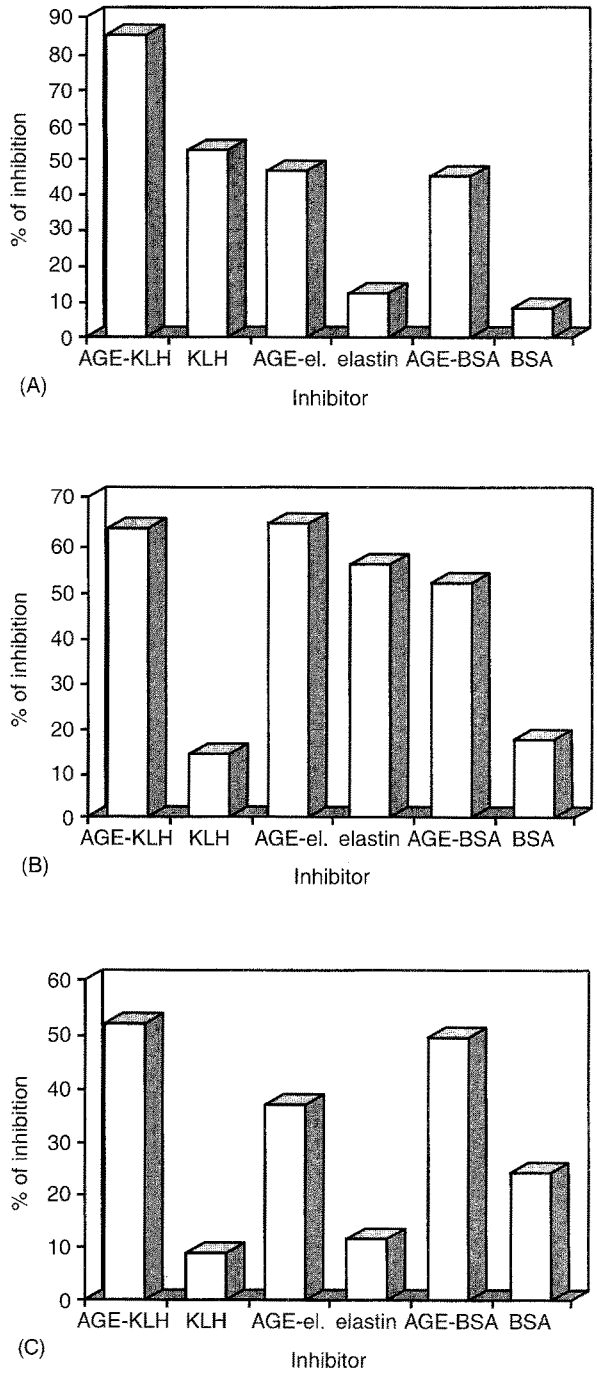

Figure 4. Results obtained by competitive ELISA for determination of the inhibitory effect of glycated and non-glycated proteins on the activity of anti-AGE-KLH immune serum. (a) inhibition of anti-AGE-KLH antibodies; (b) inhibition of anti-AGE-elastin antibodies; (c) inhibition of anti-AGE-BSA antibodies.

logous antigens (Fig. 4). The results from the competitive inhibitions of anti-AGE-KLH antibodies, anti-AGE-elastin antibodies and anti-AGE-BSA antibodies are presented separately. Data are expressed as the percentage of inhibition of the initial reactivity of the immune serum, which is assumed to be 100%.

AGE-elastin inhibited 47.1% of the activity of anti-AGE-KLH antibodies while α-elastin inhibited by only 13% (Fig. 4a). AGE-KLH and AGE-BSA inhibited 63.9% and 52.5%, respectively of the activity of anti-AGE-elastin antibodies, while KLH and BSA inhibited by 14.6% and 17.9%, respectively (Fig. 4b). AGE-elastin inhibited 37.5% of the activity of anti-AGE-BSA antibodies while α-elastin by only 12.1%. The competitive investigations of anti-AGE-KLH immune serum confirmed the existence of a population of specific anti-AGE antibodies as well as the formation of AGE-epitopes on the glycated elastin, recognized by these antibodies. The results obtained are in accordance with the commonly accepted opinion for the nature of anti-AGE antibodies [3, 4] that these antibodies recognize and react with AGE-epitopes regardless of the protein they have been formed on.

These investigations on the specificity of anti-AGE-KLH immune serum gave us a reason to develop an immunoenzyme method based on the principle of blocking ELISA for determination of Blocking factors, possibly anti-AGE antibodies, in sera of type 1 diabetic patients with non-proliferative retinopathy (Fig. 5).

Blocking factors were observed in the sera of all healthy subjects. The activity of non-blocked immune serum was $E_{492} = 0.610$ and when the healthy group was tested it was 0.41 ± 0.03 (mean $\pm$ SD). The presence of these factors which may be anti-AGE antibodies in sera of healthy subjects of 34–40 years of age, could be explained by the fact that non-enzymatic glycation of proteins has already started to stimulate antibody production. Furthermore, this finding raises questions as to when, in the course of an individual's life, does non-enzymatic glycation of long-lived proteins begin? It is a normal post-translational modification or one that occurs only in diabetes and with ageing?

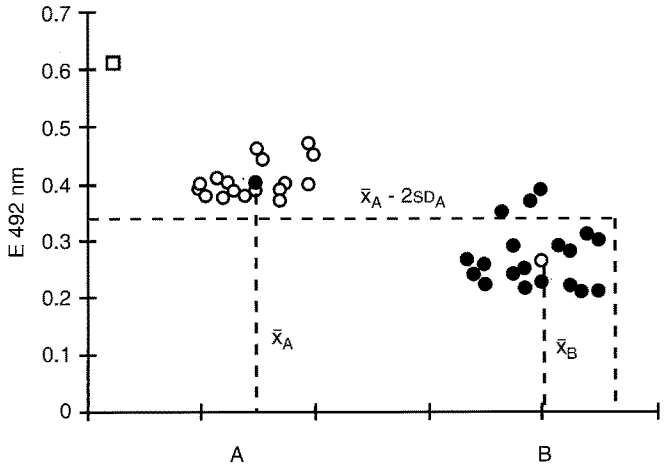

Figure 5. Results from blocking ELISA for determination of anti-AGEs antibodies. (□) Mean value in E492 of the non-blocked anti-AGE-KLH immune serum. (A) Values obtained after blocking with sera from healthy subjects; (B) values obtained after blocking with sera from diabetic patients.

It is possible that the long-lived proteins bind glucose in low concentrations, but the glycation products are under homeostatic control and therefore do not have any pathogenic effects. This suggestion is supported by the results from our previous investigation of age-related glycation of human aortic elastin [19] where AGEs were found in elastins from all age-groups, even from infants. Anti-AGE antibodies in the sera of healthy subjects, are perhaps part of a homeostatic mechanism which clears glycated structures via *in situ* destruction of glycated proteins or via opsonization of the glycated products of degradation. However, when non-enzymatic glycation of proteins is increased, the capacity of normal homeostasis seems to be inefficient. This leads to accumulation of AGEs, the pathogenic effects of which contribute to development of long-term diabetic complications and the ageing process.

With regard to the sera of the diabetic patients, we assume that those values that are equal to or under ($\bar{X}a$–2SDa) have elevated levels of factors that compete with anti-AGE antibodies. The values of 17 from 20 patients were in this range (Fig. 5B). The levels of these factors, which may be anti-AGE antibodies, are in accordance with those reported by others [1, 2] for increased non-enzymatic glycation of long-lived proteins in diabetic microangiopathy. Perhaps the excessive glycation and formation of AGEs induces increased generation of these autoantibodies. These are then involved in microvascular disease via the formation of immune complexes *in situ* and complement binding.

It must be emphasized, however, that the antigens that elicited the anti-AGE antibodies are as yet unidentified, as the guinea pig used in this study was immunised against a grossly unphysiological *in vitro* glycation mixture. Further work needs to establish the precise nature of these possible antibodies.

The role of anti-AGE antibodies in pathogenesis of diabetic complications is not clear. We have developed a specific and sensitive method which may be useful to investigate the levels and dynamics of anti-AGE antibodies in different clinical groups of diabetic patients. Such studies would help in our understanding of the pathogenic role of anti-AGE antibodies, as well as in the early diagnosis and evaluation of the severity of long-term diabetic complications.

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