Kidney International, Vol. 46 (1994), pp. 216–222

Immunological quantification of advanced glycosylation end-products in the serum of patients on hemodialysis or CAPD

PANAYIOTIS PAPANASTASIOU, LINDA GRASS, HELEN RODELA, ATHENA PATRIKAREA, DIMITRIOS OREOPOULOS, and ELEFTHERIOS P. DIAMANDIS

Division of Nephrology, Department of Medicine and Department of Clinical Biochemistry, The Toronto Hospital, Toronto Western Division, Toronto, Ontario, Canada

Immunological quantification of advanced glycosylation end-products in the serum of patients on hemodialysis or CAPD. We have developed an immunological procedure for measuring advanced glycosylation end-products (AGEs) in serum. Using this method, we measured AGEs in healthy volunteers, patients with diabetes, renal failure without treatment and in patients with renal failure, treated with hemodialysis (HD) or continuous ambulatory peritoneal dialysis (CAPD). We found that AGEs were moderately elevated in diabetics without renal failure and highly elevated in CAPD and HD patients irrespective of their glycemic status. AGE levels correlated significantly with creatinine levels but not with levels of glucose or patient age or sex. AGE levels were reduced significantly post-hemodialysis. Preliminary experiments have shown that circulating AGEs have a molecular weight of approximately 1.5 to 2.0 kDa. More studies are needed to establish if AGE measurements in serum are prognostic indicators of the complications of either diabetes or renal failure.

Reducing sugars such as glucose react non-enzymatically with the free amino groups of proteins to form stable Amadori products through Schiff base adducts. Once formed, Amadori products undergo further modifications to produce a diverse group of protein-bound moieties with cross linking properties called advanced glycosylation end-products (AGEs) [1–3]. AGEs accumulate naturally over the lifespan or under conditions of high sugar concentration such as in diabetes mellitus. Several experimental studies suggest that AGE accumulation in the body may cause structural and functional changes of proteins during aging and may play a role in the long-term complications of diabetes mellitus [4–6].

Patients with end-stage renal disease who are undergoing peritoneal dialysis include a large proportion of elderly and diabetics. We speculated that this group may be in an increased risk of accumulating AGEs because of the high concentration of glucose used as osmotic agent in the peritoneal dialysis fluid. Until now, no studies have been reported on the levels of AGEs in the serum of patients undergoing peritoneal dialysis. We examined serum AGE levels in various patient groups including peritoneal dialysis and hemodialysis patients, using an immunological procedure that we developed. Three independent reports have recently confirmed that it is possible to raise antibodies reacting with diverse AGEs, including those produced by reacting glucose with amino acids [7–9]. These reports speculate that all AGEs share a common haptenic antigenic determinant which is very useful for monitoring AGE levels in tissues and serum.

Received for publication June 30, 1993
and in revised form January 26, 1994
Accepted for publication January 31, 1994

Methods

Reagents

Bovine pancreatic ribonuclease (RNase), bovine serum albumin (BSA) and all sugars used were from Sigma Chemical Co. (St. Louis, Missouri, USA). AGE-RNase (the immunogen) was prepared by mixing 10 ml of phosphate buffer (0.2 mol/liter, pH 7.40, containing 0.5 mmol EDTA) 250 mg RNase and 1.80 g glucose, dissolving and filtering through a 0.2 μm filter and incubating for three months at 37°C (air-oven) [9]. One preparation without glucose was incubated under the same conditions as a control. The same procedure was also used to prepare an AGE-BSA solution which was used as a coating antigen in the assay, as described below. The stock AGE-BSA solution contained 50 mg of BSA/ml. After incubation, the unbound material was removed by extensive dialysis against phosphate-buffered saline.

Immunization

While female New Zealand rabbits were immunized with the AGE-RNase immunogen, which was emulsified with an equal volume of complete Fruend's adjuvant (Sigma). About 50 μl of immunogen were injected subcutaneously every two to three weeks at two different sites. Just prior to each injection, blood was drawn for antibody testing. A total of eight injections were given, and all booster injections were performed with the immunogen in incomplete adjuvant. High titers of antibodies developed after 10 to 12 weeks. The development of the AGE antibodies was monitored with a solid-phase assay described under "Procedures." The rabbit antiserum was used without further purification.

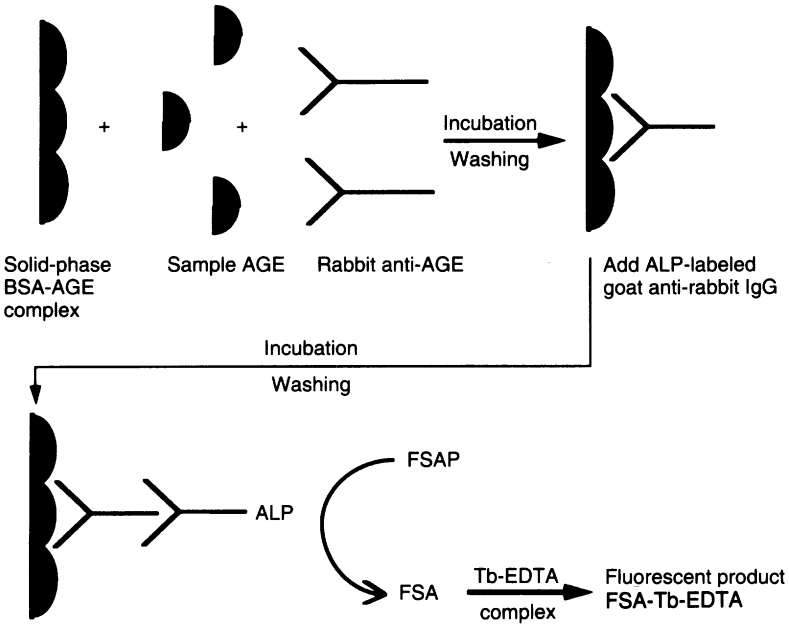

Fig. 1. *Competitive immunoassay for AGE using the immobilized antigen approach.* Abbreviations are: ALP, alkaline phosphatase; FSAP, 5-fluorosalicyl phosphate; FSA, 5-fluorosalicylate.

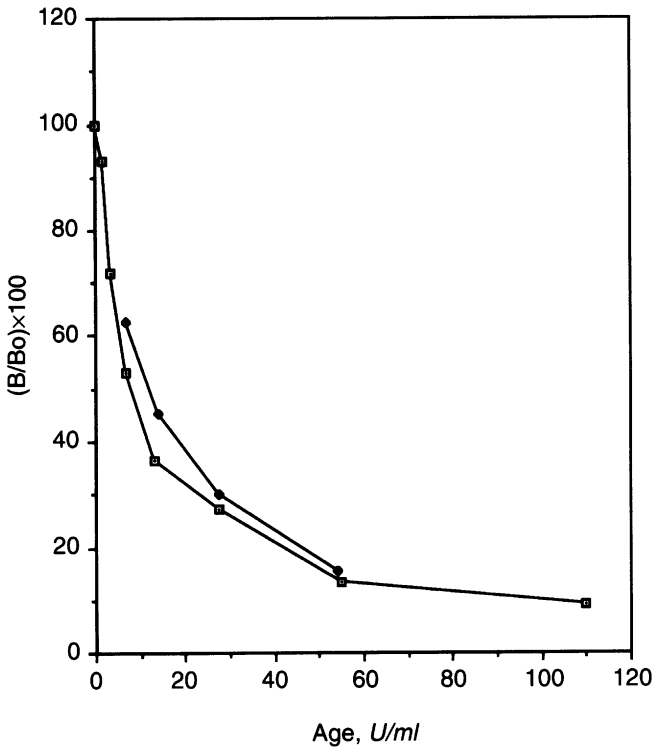

Fig. 2. *Typical calibration curve for the AGE assay* (□). The fluorescence of the zero standard was 67,409 arbitrary units and was defined as Bo. The fluorescence of all other standards was defined as B. In the same plot we present the (B/Bo) × 100 values of a serum sample that was analyzed undiluted or diluted 2-, 4- or 8-fold (◆).

Instrumentation

For measuring liquid-phase Tb^{3+} fluorescence in white microtiter wells, the CyberFluor 615™ Immunoanalyzer, a time-resolved fluorometer, was used as previously described [10, 11].

Table 1. Within-run precision of the AGE assay

AGE, *U/ml*		
Mean	SD	C.V. % (N = 12)
5.1	0.56	11.4
11.3	0.90	8.0
12.2	1.3	11.3
18.5	2.0	10.6
37.3	3.0	8.1
55.7	4.3	7.8

Abbreviations are: SD, one standard deviation; N, number of replicates.

Solutions

The enzyme substrate buffer was a 0.1 mol/liter Tris solution, pH 9.1, containing 0.1 mol NaCl and 1 mmol $MgCl_2$ per liter. The stock 5-fluorosalicyl phosphate (FSAP) substrate solution was a 10^{-2} mol/liter solution in 0.1 mol/liter NaOH. Fresh FSAP substrate working solutions were prepared just before use by 10-fold dilution of the stock in the enzyme substrate buffer. The developing solution was a 1 mol/liter Tris base solution containing 0.4 mol NaOH, 3 mmol EDTA and 2 mmol $TbCl_3 \cdot 6H_2O$ per liter (no pH adjustment). The wash solution was a 5 mmol/liter Tris buffer, pH 7.80, containing 0.5 g Tween 20 and 150 mmol NaCl per liter. The coating solution was a 0.1 mol/liter carbonate buffer, pH 9.50. The standard diluent was a 50 mmol/liter Tris buffer, pH 7.80, containing 60 g of BSA and 0.5 g of NaN_3 per liter. The assay buffer was a 50 mmol/liter Tris buffer, pH 7.80, containing 60 g BSA, 0.5 mol KCl and 10 ml normal goat serum per liter. The alkaline phosphatase-labeled goat anti-rabbit immunoglobulin, approximately 1 mg/ml, was obtained from Jackson Immunoresearch (West Grove, Pennsylvania, USA).

For the AGE assay, a series of six standard solutions with concentrations of 0, 1.7, 3.4, 6.8, 27.5 and 110 U/ml was used.

Table 2. Recovery studies

AGE, *U/ml*				
Initially present (A)	Added (B)	Total measured (C)	Recovered (D)	% Recovery[a]
10.6	14.1	29.1	18.5	131
	33.7	45.0	34.4	102
	63.2	70.6	60.0	95
5.9	14.1	22.3	16.4	116
	33.7	36.5	30.6	91
	63.2	61.8	55.9	89
8.2	14.1	28.6	20.4	144
	33.7	46.6	38.4	114
	63.2	56.9	48.7	77
8.1	14.1	24.0	15.9	113
	33.7	37.8	29.7	88
	63.2	70.0	61.9	98
			Average: 105 ± 19%	

[a] Recovered concentration D = C − A. Recovery = (D/B) × 100

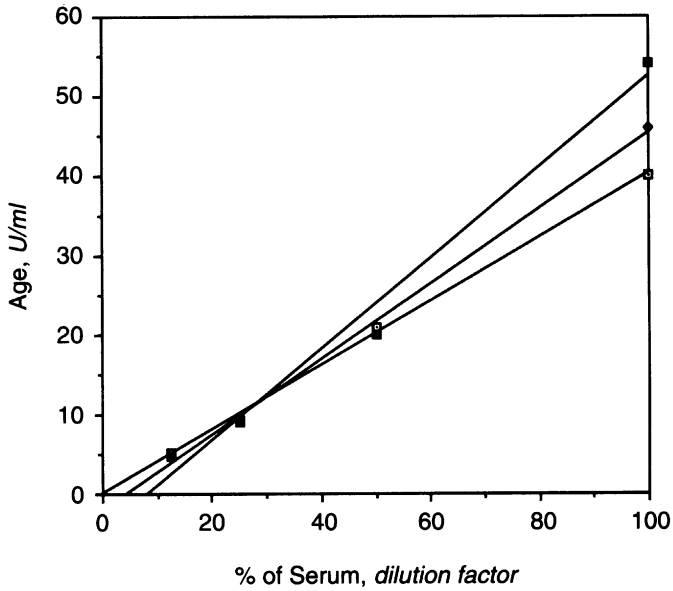

Fig. 3. *Dilution of three samples with high AGE concentration.* Samples were run undiluted (dilution factor 100) or diluted 2-fold, 4-fold and 8-fold. Linear regression analysis gave correlation coefficients of $r \geq 0.98$ in all cases.

These standards were prepared from a stock AGE-BSA solution with total protein of 50 mg/ml, equivalent to 50,000 U/ml. As also defined by others [9], one AGE unit is equivalent to 1 μg of AGE-BSA.

Patient samples and statistical analysis

Sera from various groups of patients were stored at −70°C until analysis (up to 2 months). All comparisons were determined by paired or unpaired Student's *t*-test as appropriate. All *P* values of less than 0.05 were considered to indicate statistical significance.

Immunoassay procedure

White, opaque 12-well microtiter strips (from Dynatech Labs, Alexandria, Virginia, USA) were coated with the AGE-BSA complex diluted in the coating buffer (100 μl/2.5 μg/well; overnight incubation at room temperature). Before the assay, the wells were washed six times with the wash solution, using an automated washing device. In each well 100 μl of each standard or serum sample (all in triplicate) were added to 50 μl of the rabbit anti-AGE antiserum diluted 2,000-fold in the assay buffer, incubated with shaking for two hours at room temperature and washed six times. One hundred μl/well of the alkaline phosphatase-labeled goat anti-rabbit immunoglobulin was added, diluted 5,000-fold in the assay buffer, incubated for one hour as above and washed six times. One hundred μl/well of the alkaline phosphatase substrate FSAP, diluted to 10^{-3} mol/liter in the enzyme substrate buffer was added, and incubated for 10 minutes at room temperature with shaking. Then, 100 μl/well of the developing solution was added, mixed for one minute, and the Tb^{3+}-specific fluorescence measured in the time-resolved mode as previously described [10, 11]. Data reduction was automatic through the CyberFluor 615™ Immunoanalyzer.

To monitor antibody titers during immunization, a similar assay procedure was used. The differences are as follows: (a) only the zero standard was used, and (b) the antiserum was used at various dilutions. The antibody titer was defined as the antiserum dilution that gave 50% of the maximum fluorescence count. For the assay, the antiserum titer was about 2,000.

Results

AGE formation of RNAse and BSA during the incubation periods (90 days) was monitored by fluorometry. Fluorometric measurements revealed fluorescence associated only with the AGE-RNase and AGE-BSA complexes, and not with the control mixtures of BSA and RNase with glucose and without incubation. The excitation and emission maxima of AGEs were found to be 360 and 445 nm, respectively, which is in close agreement with previously published data [12].

The immunological procedure that we developed was similar to the one described by Makita et al [9], but for detection, we used a methodology based on enzymatically amplified time resolved fluorometry with terbium chelates, as previously described [10, 11]. In our assay (Fig. 1) immobilized AGE (AGE-BSA) competed with the AGE in the serum sample or standards, for binding to a limited amount of anti-AGE rabbit antibodies. The degree of binding of the antibody to the solid-phase was inversely related to the amount of AGE in the sample. Antibody binding was quantified with a goat anti-rabbit antibody covalently linked to alkaline phosphatase (ALP). The ALP substrate used was FSAP, which was converted to 5-fluorosalicylate (FSA). FSA is able to form a ternary fluorescent complex with Tb-EDTA of a long fluorescence lifetime [13].

A typical calibration curve for the AGE assay is shown in Figure 2. This assay measured AGE up to 110 U/ml and had a detection limit of approximately 1 U/ml. The precision of the assay at various AGE concentrations is shown in Table 1. Recovery experiments done by spiking patient sera with AGE-BSA are summarized in Table 2. The possible interference of simple sugars, that is, glucose, xylitol, dulcitol and galactose, was tested at concentrations as high as 60 mmol/liter and of heparin at contentrations up to 1000 U/ml. None interfered to

Table 3. Serum levels of AGE in various groups of patients

Group	*N*	Age, *years*		Glucose *mmol/liter*		Creatinine *μmol/liter*		AGE, *U/ml*			
		Mean	SD[d]	Mean	SD	Mean	SD[d]	Mean	SD[d]	Minimum	Maximum
Normal subjects	18	47	16	—	—	—	—	7.8	2.3	3.9	11.7
Diabetics, no renal failure	24	56	14	11.0	4.0	123	45	15.0	5.5	4.6	28.4
NIDDM	15	56	13	11.0	4.3	128	49	16.2	5.2	8.8	28.4
IDDM	9	57	16	11.0	3.6	114	32	12.8	5.7	4.6	18.7
Renal failure, non-treated	9	67	17	4.3	2.0	367	225	25.4	14.3	16.0	66.2
CAPD	26	51	18	8.6	5.0	762	360	29.5	13.3	11.6	60.2
Non-diabetics	14	53	21	5.8	1.9	885	262	31.3	16.6	11.6	60.2
NIDDM	3	39	13	8.4	5.4	585	294	25.7	6.4	20.1	32.7
IDDM	9	53	16	13.0	5.5	817	277	28.2	9.0	16.1	43.5
Hemodialysis patients	21	61	14	7.0	3.6	534	330	24.0	9.6	9.7	49.0
Non-diabetics-pre[a]	6	57	19	5.1	0.4	951	165	38.1	7.2	30.3	49.0
Non-diabetics-post[b]	6	57	19	4.3	0.5	394	63	16.3	5.8	9.7	26.1
NIDDM-pre	4	68	9	9.2	1.6	674	72	32.7	4.8	19.3	37.2
NIDDM-post	4	68	9	6.3	1.4	274	32	18.2	5.5	11.7	23.5
NIDDM-R[c]	4	72	8	11.0	5.3	279	67	22.0	6.9	11.8	29.6
IDDM-pre	1	53	—	10.0	—	567	—	31.3	—	—	—
IDDM-post	1	53	—	10.4	—	337	—	25.3	—	—	—
IDDM-R[c]	6	55	13	5.4	1.2	582	404	25.0	4.8	17.9	31.2

Abbreviations are: *N*, number of patients; NIDDM, non-insulin dependent diabetes mellitus; IDDM, insulin dependent diabetes mellitus; CAPD, continuous ambulatory peritoneal dialysis.
[a] Sample was taken before hemodialysis
[b] Sample was taken after hemodialysis
[c] R, random samples between hemodialysis treatments
[d] SD, one standard deviation

any measurable degree. Three samples with high AGE concentration were analyzed undiluted or diluted two-, four- or eightfold using a 6% BSA solution as diluent. The results are shown in Figure 3. The near-linear relationship between AGE concentration and dilution further suggested that the AGEs in serum and AGE-BSA share a common antigenic determinant [7–9]. A serum sample was plotted, diluted either two-, four- or eightfold along with the standard curve (Fig. 2). For this experiment, the AGE concentration in the undiluted sample was calculated from the standard curve and the fluorescence of the dilutions plotted, expressed as (B/Bo) × 100, versus the concentration of AGE as predicted by the dilution factor. The two curves, obtained with AGE-BSA and diluted serum, are closely related (Fig. 2).

The results of AGE analysis in various groups of patients are summarized in Table 3. The mean values of AGEs in normal subjects and diabetics with normal renal function were statistically different ($P < 0.001$). Patients with renal failure without diabetes and patients treated with continuous ambulatory peritoneal dialysis (CAPD) or hemodialysis had highly elevated serum levels of AGE, irrespective of their glycemic status. Comparison of various groups of patients with respect to AGE concentration, by the Student's *t*-test, revealed highly significant differences between the following groups: normals versus diabetics; normals versus NIDDM; normals versus IDDM; normals versus CAPD (all patients); normals versus hemodialysis (all patients); diabetics versus CAPD (all patients); and diabetics versus hemodialysis (all patients). In all cases the *P* values were <0.004. In contrast, comparison of the following groups with respect to AGE concentration gave no statistically significant differences: CAPD versus hemodialysis ($P = 0.13$); renal failure patients (non-treated) versus CAPD ($P = 0.56$); renal failure patients (non-treated) versus hemodialysis ($P = 0.72$); CAPD, non-diabetics versus CAPD, NIDDM ($P = 0.59$); CAPD, non-diabetics versus CAPD, IDDM ($P = 0.62$).

The results of whether AGE concentration was associated with serum glucose or creatinine levels, sex or age are summarized in Figure 4. No correlation or association existed between AGE levels and glucose, sex or age. However, there was a strong correlation between AGE levels and creatinine in both the CAPD and hemodialysis patient groups. The variation of AGE levels during hemodialysis was also examined. For these studies, predialysis and postdialysis AGE and creatinine levels were compared in three groups of patients: non-diabetics, NIDDM and IDDM (Fig. 5). In all cases there was a highly significant drop in AGE levels after hemodialysis, which suggests that the AGEs are relatively effectively cleared.

Nine patients were studied to determine if the AGE accumulation in renal failure patients was the result of the hemodialysis or CAPD treatment or if it occurred before the treatment was initiated (Table 3). These patients had renal failure but blood sampling for AGE analysis was obtained before the initiation of therapy. The AGE levels were elevated in these patients, confirming that the AGE accumulation was not a consequence of the treatment.

Molecular weight identification of AGE

One ml of a patient serum sample with high AGE concentration was dialyzed against 5 liters of saline overnight using a dialysis membrane with a cutoff level of 12,000 Da. Analysis of the AGE levels in the serum, after dialysis, showed an 80% decrease, suggesting that AGE was effectively dialyzed. Similar results were obtained when the sample was ultrafiltered using

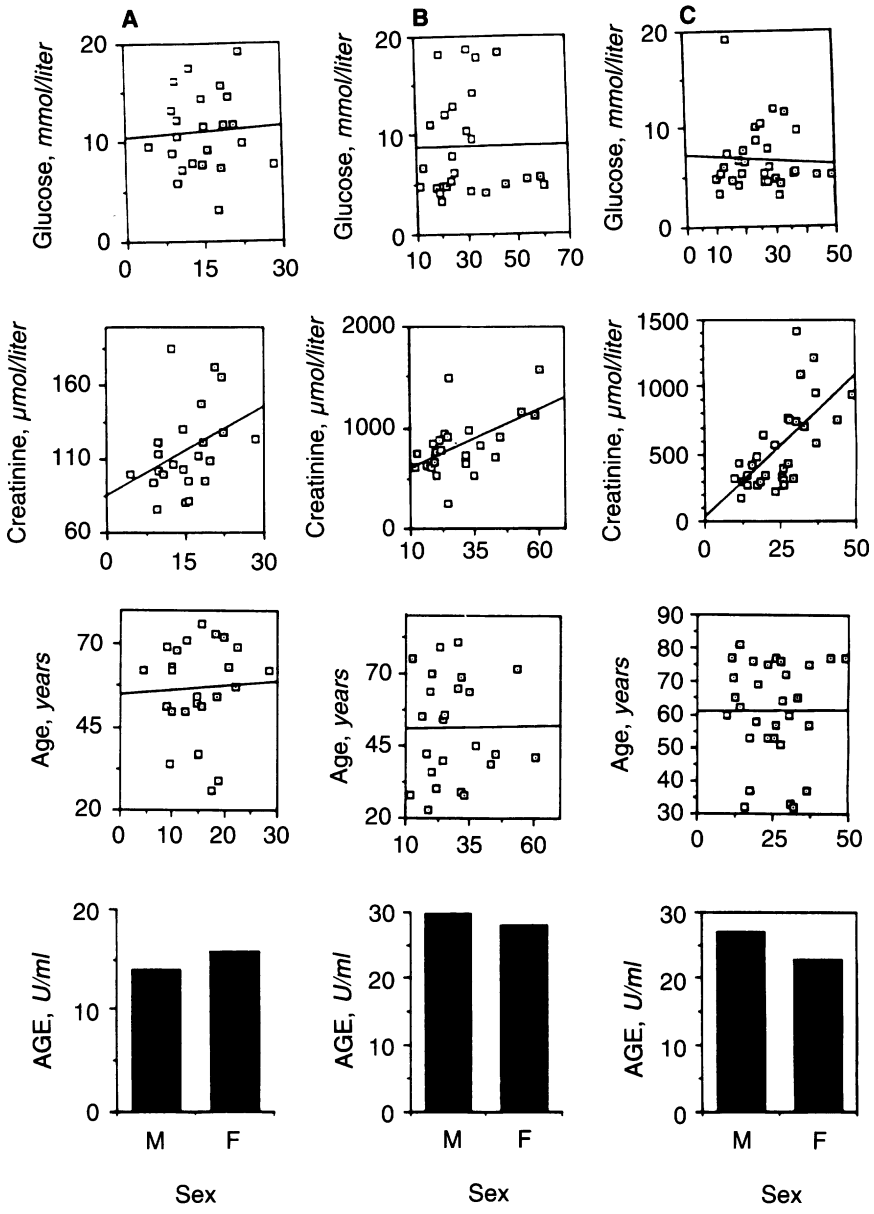

Fig. 4. *Correlations of AGE levels with glucose, creatinine, age and sex.* Left row (**A**) represents the group of diabetic patients. Middle row (**B**) are CAPD patients and right row (**C**) hemodialysis patients. Statistically significant correlations were observed only with the creatinine levels. The correlation coefficients r were 0.38 for A, 0.55 for B and 0.66 for C. M = male; F = female.

Amicon filters and centrifugation. To further identify the molecular weight of AGE, one serum sample was chromatographed through a Bio-Sil SEC-250 column, 600 mm × 7.5 mm (Bio-Rad Laboratories, Richmond, California, USA) using a mobile phase of 0.1 mol/liter Na_2SO_4-0.1 mol/liter NaH_2PO4, pH 6.80, at a flow rate of 0.5 ml/min. After injecting 1 ml sample, 0.5 ml fractions were collected and analyzed for AGEs. The column was calibrated with a molecular weight standard solution from BioRad. The results are shown in Figure 6. Clearly, AGEs elute at a peak corresponding to a molecular weight of 1500 to 2000 daltons.

Discussion

Advanced glycosylation end-products are candidate pathogenetic factors of the long-term complications of diabetes. Extensive clinical studies on AGEs, especially with assessment of the serum levels, are lacking partially because their measurement is difficult and commercial assays are not available. This paper describes an immunological assay for AGEs in serum, based on a rabbit antibody that was raised with AGE-RNase as immunogen, as described by Makita et al [9]. AGE formation was followed by fluorometric procedures. Our competitive assay utilizes a solid-phase AGE-BSA complex and a detection methodology based on time-resolved fluorometry with terbium chelates as labels [10–12]. The assay was found to be precise, accurate and easy to perform. No interference by simple sugars or heparin was observed, in accordance with reports by three other groups [7–9] which suggest that anti-AGE antibodies, raised following the procedures described, react with an epitope specific and common to all AGEs. This epitope is not present in early glycosylated products or synthetic model AGE molecules [9].

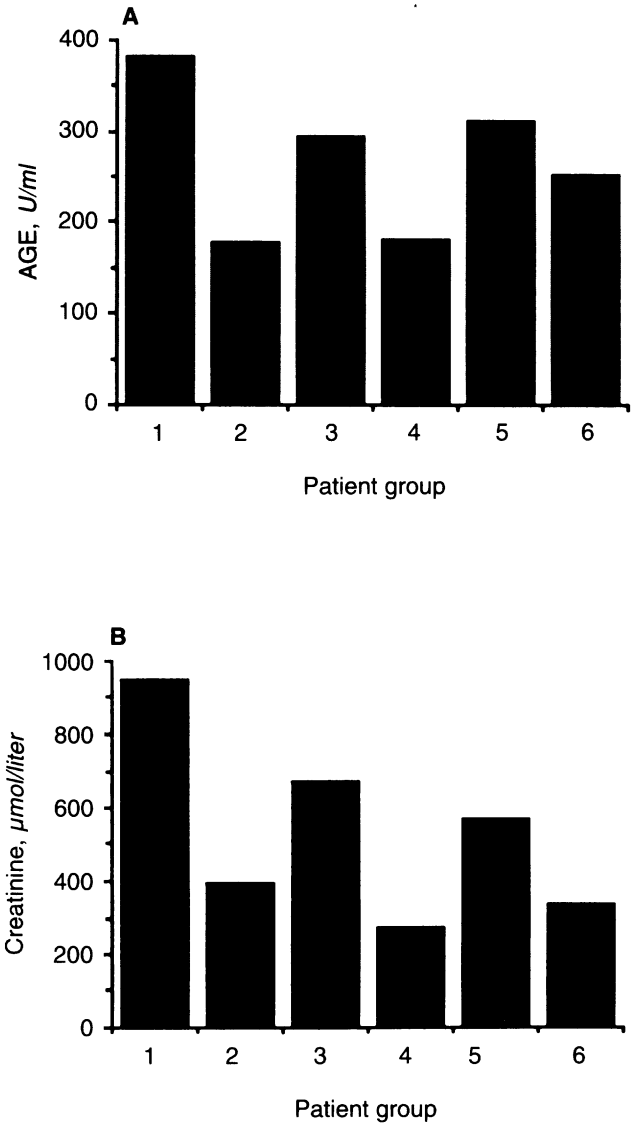

Fig. 5. *Pre- and post-hemodialysis levels of AGE* (**A**) *and creatinine* (**B**) *in patients with renal failure: 1, 3, 5, pre-dialysis; 2, 4, 6, post-dialysis.* Patient groups were: non-diabetics (1, 2; $N = 6$); NIDDM (3, 4; $N = 4$) and IDDM (5, 6; $N = 1$). The pre- and post-dialysis values were compared by the t-test; the value of P in each case was: $P = 0.001$ for AGE and <0.001 for creatinine (non-diabetics); $P = 0.036$ for AGE and 0.001 for creatinine (NIDDM). No statistical comparison was done in the patient with IDDM.

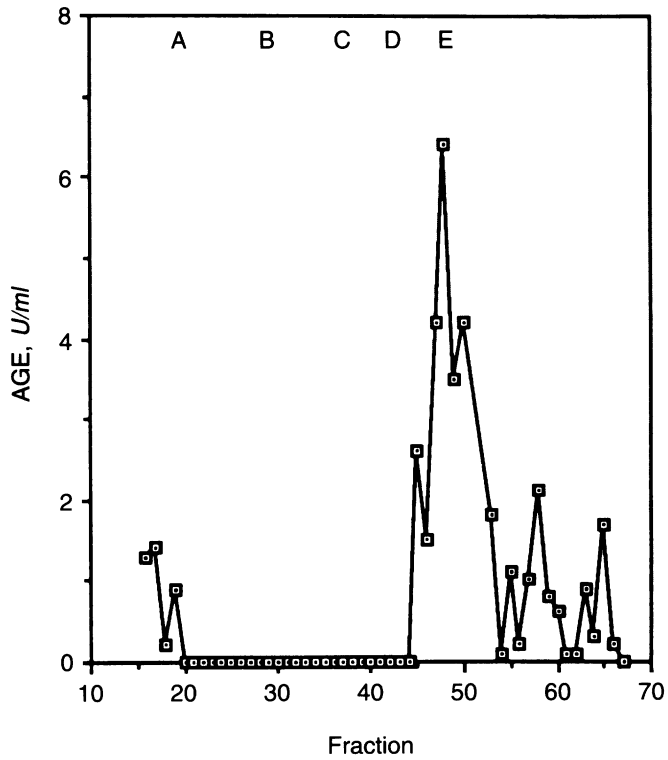

Fig. 6. *High performance liquid chromatography of a patient serum sample with high AGE concentration.* The position of the molecular weight standards is shown by **A** (660 KDa), **B** (158 KDa), **C** (44 KDa), **D** (17 KDa) and **E** (1.4 KDa). AGEs elute at a molecular weight of approximately 1.5 to 2.0 KDa.

Our clinical studies have clearly shown that diabetic patients without renal failure have elevated AGE levels in serum (15.0 U/ml vs. 7.80 U/ml in normals, $P < 0.001$) in accordance with a recent report [9]. We also found much higher circulating AGE levels in the serum of hemodialysis patients, also in agreement with previously published data [14–16]. CAPD patients were further demonstrated to have highly elevated AGE levels in their serum. AGE elevation in the serum of CAPD and hemodialysis patients exists irrespective of the presence of diabetes. The strong correlation of AGE levels with serum creatinine suggests that AGEs accumulate during renal failure and probably reach saturating levels in serum irrespective of the presence of diabetes or not. AGEs are partially removed during hemodialysis. No association was found between AGE levels and sex, age or serum glucose levels. Other studies have shown that AGEs pre-exist in patients with renal failure and are not the consequence of the therapy.

The molecular weight of the circulating AGEs is around 1500 to 2000 Da as found by high performance liquid chromatographic studies. Currently, it is believed that serum AGEs are peptides in nature but this has not yet been confirmed, nor is their precise structure known. We are currently examining if the measurement of AGEs in serum by the immunological assay described has clinical significance in predicting the long-term complications of diabetic and non-diabetic nephropathy.

Acknowledgments

This study was supported by grants to E.P. Diamandis from the Medical Research Council of Canada and the University Research Incentive Fund (URIF) of the Ministry of Colleges and Universities of the Province of Ontario.

Reprint requests to Eleftherios P. Diamandis, Department of Clinical Biochemistry, Toronto Western Division, TTH, 399 Bathurst Street, Toronto, Ontario M5T 2S8, Canada.

References

1. Lee AT, Cerami A: Role of glycation in aging. *Ann NY Acad Sci* 663:63–70, 1992
2. Bucala R, Cerami A: Advanced glycosylation: Chemistry biology, and implications for diabetes and aging. *Adv Pharmacol* 23:1–34, 1992

3. VLASSARA H, BROWNLEE M, CERAMI A: Nonenzymatic glycosylation of peripheral nerve protein in diabetes mellitus. *Proc Natl Acad Sci USA* 78:5190–5192, 1981
4. BROWNLEE M, VLASSARA H, CERAMI A: Nonenzymatic glycosylation and the pathogenesis of diabetic complications. *Ann Intern Med* 101:527–537, 1984
5. MANDEL SS, SHIN DH, NEWMAN BL, LEE JH, LUPOVITCH A, DRAKES GH: Glycosylation *in vivo* of human lens capsule (basement membrane) and diabetes mellitus. *Biochem Biophys Res Commun* 117:51–56, 1983
6. MONNIER VM, VISHWANATH V, FRANK KE, ELMETS CA, DAUCHOT P, KOHN RR: Relation between complications of type I diabetes mellitus and collagen-linked fluorescence. *N Engl J Med* 314:403–408, 1986
7. NAKAYAMA H, TANEDA S, KUNAJIMA S, KUNAJIMA S, AOKI S, KURODA Y, MISAWA K, NAKAGAWA S: Production and characterization of antibodies to advanced glycation products of proteins. *Biochem Biophys Res Commun* 162:740–745, 1989
8. HORIUCHI S, ARAKI N, MORINO Y: Immunochemical approaches of characterize advanced glycation end products of the Maillard reactions. *J Biol Chem* 266:7329–7332, 1991
9. MAKITA Z, VLASSARA H, CERAMI A, BUCALA R: Immunochemical detection of advanced glycosylation end products *in vivo*. *J Biol Chem* 267:5133–5138, 1992
10. CHRISTOPOULOS TK, DIAMANDIS EP: Enzymatically amplified time-resolved fluorescence immunoassay with terbium chelates. *Anal Chem* 64:342–346, 1992
11. PAPANASTASIOU-DIAMANDI A, CHRISTOPOULOS TK, DIAMANDIS EP: Ultrasensitive thyrotropin immunoassay based on enzymatically amplified time-resolved fluorescence with a terbium chelate. *Clin Chem* 38:545–548, 1992
12. EDELSTEIN D, BROWNLEE M: Mechanistic studies of advanced glycosylation end products inhibition by aminoguanidine. *Diabetes* 41:26–29, 1992
13. EVANGELISTA RA, POLLAK A, GUDGIN-TEMPLETON EF: Enzyme-amplified lanthanide luminescence for enzyme detection in bioanalytical assays. *Anal Biochem* 197:213–224, 1991
14. MAKITA Z, RADOFF S, RAYFIELD EJ, YANG Z, SKOLNIK E, DELANEY V, FRIEDMAN EA, CERAMI A, VLASSARA H: Advanced glycosylation end products in patients with diabetic nephropathy. *N Engl J Med* 325:836–842, 1991
15. STEFFES MW, MANER MS: Toward a basic understanding of diabetic complications. *N Engl J Med* 325:883–884, 1991
16. RADOFF S, MAKITA Z, VLASSARA H: Radioreceptor assay for advanced glycosylation end products. *Diabetes* 40:1731–1738, 1991