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# The serum concentration of the advanced glycation end-product $N^{\epsilon}$-(carboxymethyl)lysine is increased in uremia

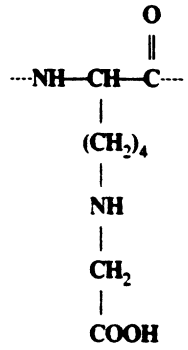
## *Technical Note*

**THORSTEN P. DEGENHARDT, LINDA GRASS, SHARANYA REDDY, SUZANNE R. THORPE, ELEFTHERIOS P. DIAMANDIS, and JOHN W. BAYNES**

*Department of Chemistry and Biochemistry, and Department of Ophthalmology, University of South Carolina, Columbia, South Carolina, USA; the Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, and the Department of Clinical Biochemistry, University of Toronto, Ontario, Canada*

**The serum concentration of the advanced glycation end-product $N^{\epsilon}$-(carboxymethyl)lysine is increased in uremia.** Advanced glycation end products (AGEs) such as pentosidine and $N^{\epsilon}$-(carboxymethyl)lysine (CML) have been traditionally quantified by HPLC or gas chromatography—mass spectrometry (GC/MS). Enzyme-linked immunosorbent assays (ELISA) have been introduced as a convenient alternative to simplify the detection and measurement of AGEs in proteins and tissues, but some of these studies are limited by the lack of information on the structure of the epitopes recognized by antibodies to AGE-proteins. In this work we demonstrate that an antibody used in a previous study, reporting increased levels of AGEs in patients with diabetes or on continuous ambulatory peritoneal dialysis (CAPD) and hemodialysis (HD), recognizes CML as its major epitope. We also show that there is a significant correlation between the concentration of AGEs in serum measured by ELISA and a GC/MS assay for CML in serum proteins. Both analyses yielded comparable results, with patients on CAPD and HD having about threefold higher AGE- or CML-concentrations in their serum. Our data suggest that ELISA assays for CML should be useful for the clinical measurement of AGEs in serum proteins.

Advanced glycation end-products (AGEs) are formed following glycation of protein during the Maillard reaction and are implicated in the development of long-term, chronic complications of diabetes [1, 2], atherosclerosis [3], hemodialysis-associated amyloidosis [4, 5] and neurodegenerative diseases [6, 7; reviewed in 8]. AGEs such as pentosidine [9, 10] and $N^{\epsilon}$-(carboxymethyl)lysine (CML) [11, 12] have been traditionally quantified by sophisticated instrumental techniques, such as reversed phase HPLC with fluorescence detection or selected ion monitoring gas chromatography—mass spectrometry (SIM-GC/MS), methods that are not readily applied to large numbers of samples in a clinical laboratory setting. During the last few years immunological techniques have been introduced to simplify the detection and measurement of AGEs in proteins, including immunohistochemical detection of AGEs in renal basement membrane [13], in Alzheimer's plaque in brain [6], in amyloid deposits in hemodialysis patients [4], and in



CML

atherosclerotic plaque [14], and ELISA assays for quantifying AGEs in lens proteins [15], serum proteins [16–18] and skin collagen [19, 20]. One limitation of these studies is that the epitope recognized by the antibodies was unknown at the time the studies were published, and, in some cases, is still unknown [17, 20]. Reddy et al [21] reported, however, that the glycoxidation product CML was a major epitope recognized by anti-AGE polyclonal antibodies. Ikeda et al [22] later confirmed the CML specificity of their monoclonal antibody, 6D12, which had been used for immunohistochemical detection of AGEs in atherosclerotic plaque [14] and in ELISA assays for quantifying AGEs in collagen [19] and lens proteins [15]. Fu et al [23] also reported recently that CML could be formed as a by-product of lipid peroxidation reactions, suggesting that CML might be a general biomarker of oxidative stress and oxidative modification of proteins by products of oxidation of both carbohydrates and lipids.

In a recent publication in this journal, some of us [24] have applied a polyclonal antibody (pDia1), prepared against AGE-proteins to detect and quantify AGEs in serum and, using this antibody, detected an increase of AGEs in serum of diabetic patients and in renal failure patients on hemodialysis (HD) and

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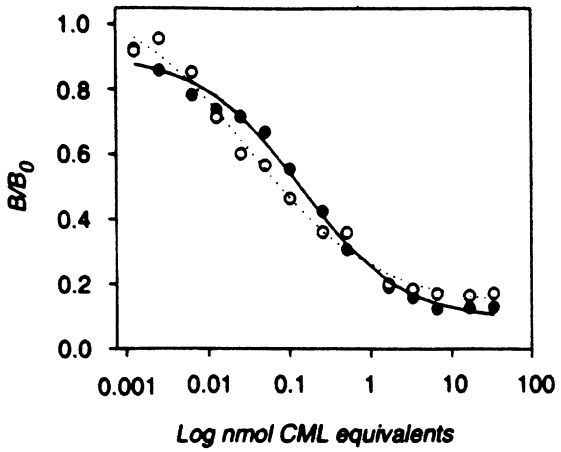


**Fig. 1. Inhibition of anti-AGE antibody recognition of AGE-RbSA by CML-BSA (○) and AGE-RbSA (●). Sample concentrations are expressed in CML equivalents, measured by amino acid analysis. Similar results were obtained for three other antibody preparations (pDia2, pDia3 and pSR1; data not shown). Data are expressed as ratios of response with competitor (B) to controls without competitor ($B_0$).**

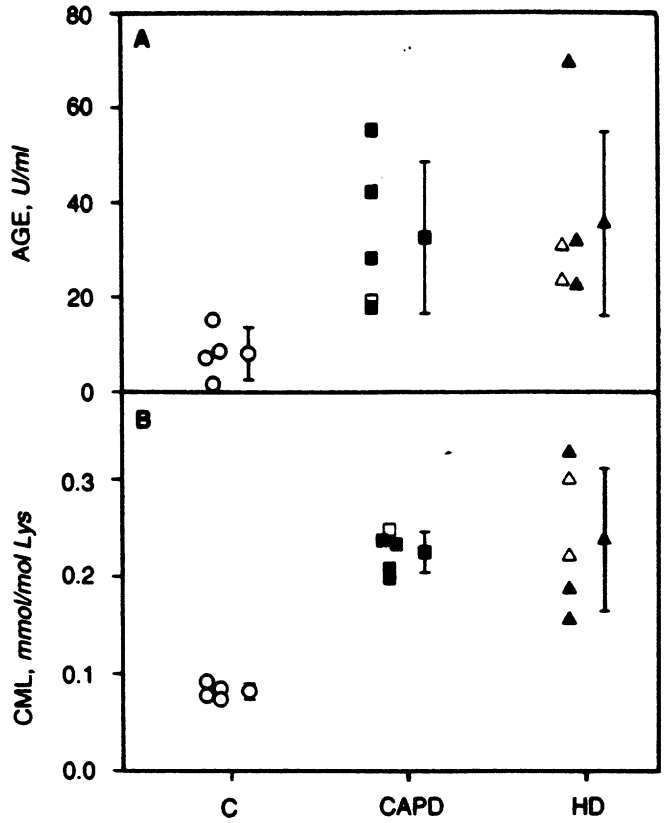


**Fig. 2. AGE and CML concentration in serum of control, CAPD and HD patients. (*A*) AGE-content (one AGE-unit is defined as the equivalent to 1 μg of AGE-BSA) measured by competitive ELISA using time resolved fluorescence detection [24]. CAPD versus C: $P < 0.02$; HD versus C: $P < 0.03$. (*B*) CML-content measured by SIM-GC/MS [26]. CAPD versus C: $P < 0.0001$; HD versus C: $P < 0.02$. Data are mean ± SD.**

continuous ambulatory peritoneal dialysis (CAPD). Similar results were previously reported by Makita et al [17] and Nakayama et al [25], but in none of these reports was the specificity of the antibody known. In the present study we demonstrate that PDia1 recognizes CML in serum proteins, and that there is a good correlation between ELISA and SIM-GC/MS assays for CML in serum proteins.

## METHODS

### Materials

$N^{\epsilon}$-(carboxymethyl)lysine (CML)-derivatized bovine serum albumin (CML-BSA) was prepared by reaction of glyoxylic acid with BSA in the presence of $NaBH_3CN$, as described previously [21]. AGE-rabbit serum albumin (AGE-RbSA) was prepared by incubation of rabbit serum albumin with 1 mol/liter glucose in phosphate buffer [21]. The CML content of the proteins was determined by amino acid analysis following acid hydrolysis, as described previously [21]. AGE-RbSA contained 17 mol CML per mol protein, CML-BSA was modified with 30 mol CML per mol protein. Polyclonal antibodies pDia1, pDia2 and pDia3 were prepared by immunization of rabbits with AGE-RNase, prepared by incubating RNase in phosphate buffer containing 1 mol/liter glucose for three months at 37°C, as described previously [24].

Serum samples were collected from four control, five CAPD and five HD patients at the Division of Nephrology (Toronto Hospital, Western Division), according to standard and approved procedures. Four of the CAPD and three of the HD patients were diabetic.

### Immunoassay procedures

Competitive ELISA assays were conducted in Costar (Cambridge, MA, USA) multiwell polystyrene plates, coated with 2.5 μg of AGE-RbSA in 0.05 mol/liter carbonate buffer pH 9.6 for two hours at 37°C [21]. After washing the wells seven times with phosphate buffered saline (PBS), pH 7.4, containing 0.05% Tween 20, wells were blocked with 300 μl of 1% ovalbumin in PBS-Tween buffer. In the competition step, 200 μl of the competition mixture of CML-BSA or AGE-RbSA [21] as competitor and a 1:8000 dilution of anti-rabbit antiserum (PDia1, 2 or 3; antibodies were prepared with the same immunization procedure, but in three different animals) were added to the wells and incubated for two hours at 37°C. Wells were washed, then incubated with a 1:5000 dilution of horseradish peroxidase-linked goat anti-rabbit immunoglobulin (Bio-Rad, Hercules, CA, USA) for one hour at 37°C and developed with 200 μl of substrate solution containing 25 mg of 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Sigma Chemicals Inc., St. Louis, MO, USA) and 30 μl of 30% hydrogen peroxide per 100 ml 0.1 mol/liter sodium citrate buffer pH 4.5, as previously described [21]. Absorbance was measured at 410 nm after approximately 30 minutes of incubation time.

ELISA assays for the AGE content of serum samples were conducted as described previously, using $Tb^{3+}$-fluorescence for detection [24].

### SIM-GC/MS assays for CML in serum proteins

Serum samples (70 μl) were reduced in 500 μl of 100 mmol/liter sodium borohydride in 0.2 mol/liter sodium borate buffer, pH 9.1, for four hours at room temperature and dialyzed against deionized water overnight with two water exchanges. The serum samples were then delipidated by addition of 12 volumes of

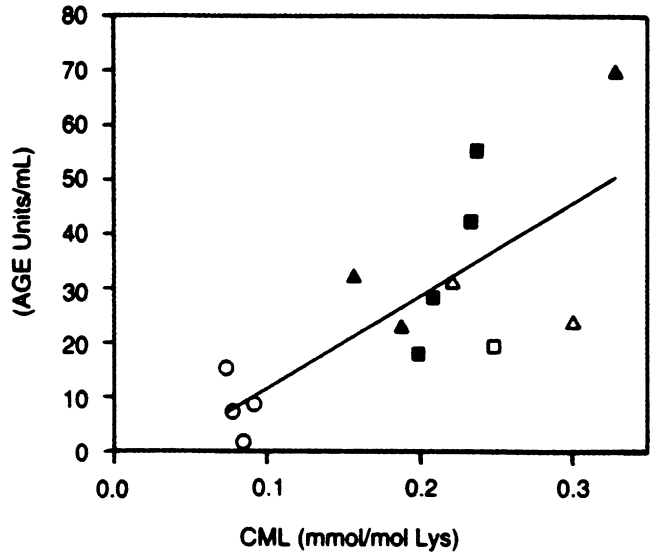


**Fig. 3. Correlation between AGE and CML concentrations in serum of controls, CAPD and HD patients.** AGE-ELISA and GC/MS data are from Figure 1. Symbols are: (○) controls; (■) CAPD; (▲) HD patients. Open symbols indicate samples from non-diabetic patients. The line drawn is a linear least squares fit to the data ($r$ = 0.75). Pearson Product Moment non-parametric analysis yielded $P < 0.002$.

water:methanol:water-saturated diethylether (1:3:8), followed by centrifugation at 1000 rpm for 15 minutes in a benchtop centrifuge. The precipitate was resuspended in 1 ml of water by vortexing, followed by reprecipitation with methanol/diethylether (3:8) and centrifugation. After drying of the aqueous phase *in vacuo* (Savant Instruments, Farmingdale, NY, USA), internal standards (17 nmol $d_8$-lysine and 1 nmol $d_4$-CML) were added and the samples were hydrolyzed for 24 hours in 2 mL of 6 mol/liter HCl at 110°C. Amino acids were analyzed as trifluoroacetyl methyl ester (TFAME) derivatives on an HP series 6980 gas chromatograph—mass spectrometer, as described previously [26].

### Statistical analyses

Data are expressed as mean ± SD. $P$ values were calculated using the Student's $t$-test. Correlation coefficients were determined by a Pearson Product Moment non-parametrical analysis using SigmaStat™ V1.0 for Windows (Jandel Scientific, Ventura, CA, USA).

## RESULTS

To evaluate the antigenic specificity of pDia1, used in the original study, we compared the effectiveness of CML-BSA and AGE-RbSA as inhibitors of recognition of AGE-RbSA coated on microtiter plates. As shown in Figure 1, CML-BSA and AGE-RbSA were equally effective in competing with immobilized AGE-RbSA, when the proteins were compared on the basis of their CML content. Half-maximal competition was observed for both proteins at approximately 0.1 nmol CML-equivalents, amounting to 3 nmol of CML-BSA and 5.9 nmol of AGE-RbSA, respectively. At higher concentrations, both proteins fully inhibited recognition of immobilized AGE-RbSA, suggesting specificity of the antibody for the same epitope, CML. Underivatized proteins yielded less than a 10% decrease in $B/B_0$ at protein concentrations comparable to those used in the experiments in Figure 1.

Figure 2 shows the results of analyses of serum proteins from control, CAPD and HD patients, assayed for their AGE content by ELISA (Fig. 2A) and CML content by SIM-GC/MS (Fig. 2B) techniques. By both methods, patients on CAPD or HD have about threefold higher concentrations of AGEs in their serum, compared to healthy controls. There was no significant difference in serum levels of CML by either assay in CAPD, compared to HD patients. Figure 3 shows that there was a good correlation between the AGE content of serum proteins measured by the ELISA and SIM-GC/MS assays.

## DISCUSSION

In the present study we have determined that polyclonal anti-AGE antibody PDia1 recognizes the epitope CML in AGE-proteins (Fig. 1). We have also demonstrated that the AGE content of serum proteins, measured by an ELISA assay using PDia1 antibody, correlates with the CML content of the protein measured by SIM-GC/MS (Fig. 3), and that by both methods there are similar, approximately threefold increases in AGEs or CML in serum proteins from CAPD and HD patients. These data constitute the first demonstrated correlation between ELISA and SIM-GC/MS assays for measurement of CML in serum proteins and confirm that the specific AGE, CML, is significantly increased in serum proteins of dialysis patients. Similar results were obtained with three other antibodies (pDia2 and pDia3, unpublished data; pSR1 [21]), supporting the previous identification of CML as a major epitope recognized by polyclonal anti-AGE antibodies [21]. Differences in the avidity with which CML is recognized on different proteins may explain differences between results of the ELISA and SIM-GC/MS assays for CML, which are apparent as deviations from the line corresponding to the least squares fit in Figure 3.

The identification of CML as the antigen recognized by the pDia antibodies validates the use of ELISA assays in future studies on the measurement of serum AGEs. The ELISA procedure is clearly more useful in a clinical setting for assessing the relationship between AGEs and vascular complications of diabetes and renal disease. However, in our hands, the results obtained with the ELISA procedure are sensitive to variations in sample preparation, such as plasma versus serum, EDTA plasma versus heparin plasma, native versus alkaline treated plasma, and vary with the type of tissue or fluid used, so that choice of an appropriate standard and validation of results of ELISA assays by the SIM-GC/MS are essential for inter-laboratory comparisons.

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*Reprint requests to Dr. John W. Baynes, Department of Chemistry and Biochemistry, University of South Carolina, Columbia, South Carolina 29208, USA.*
*E-mail: Baynes@psc.sc.edu*

## APPENDIX

Abbreviations are: AGE, advanced glycation end-product; AGE-RbSA, AGE-rabbit serum albumin; ABTS, 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); CML-BSA, CML-derivatized bovine serum albumin; CAPD, continuous ambulatory peritoneal dialysis; HD, hemodialysis;

CML, $N^{\epsilon}$-(carboxymethyl)lysine; SIM-GC/MS, selected ion monitoring gas chromatography–mass spectrometry.

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