J. Biochem. **136**, 831–837 (2004)
DOI: 10.1093/jb/mvh193

Conventional Antibody against N^{ε}-(Carboxymethyl)Lysine (CML) Shows Cross-Reaction to N^{ε}-(Carboxyethyl)Lysine (CEL): Immunochemical Quantification of CML with a Specific Antibody

Wakako Koito[1], Tomohiro Araki[2], Seikoh Horiuchi[1] and Ryoji Nagai[1,*]

[1]Department of Medical Biochemistry, Faculty of Medical and Pharmaceutical Sciences, Kumamoto University, Honjo 1-1-1, Kumamoto 860-8556; and [2]Faculty of Agriculture, Kyushu Tokai University, Kumamoto

Received August 17, 2004; accepted October 9, 2004

Immunological strategies for the detection of N^{ε}-(carboxymethyl)lysine (CML), one of the major antigenic structures of advanced glycation end products (AGE), are widely applied to demonstrate the contribution of CML to the pathogeneses of diabetic complications and atherosclerosis. Recent studies have indicated that methylglyoxal (MG), which is generated intracellularly through the Embden-Meyerhof and polyol pathways, reacts with proteins to form MG-derived AGE structures such as N^{ε}-(carboxyethyl)lysine (CEL). In order to accurately measure the CML contents of the proteins by means of an immunochemical method, we prepared CML-specific antibodies since conventionally prepared polyclonal anti-CML antibody and monoclonal anti-CML antibody (6D12) cross-reacted with CEL. To prepare polyclonal CML-specific antibody, CML-keyhole limpet hemocyanin (CML-KLH) were immunized with rabbit and CEL-reactive antibody was removed by CEL-conjugated affinity chromatography. Monoclonal antibody specific for CML (CMS-10) was obtained by immunization with CML-KLH, followed by successive screening according to CML-bovine serum albumin (CML-BSA)–positive but CEL-BSA-negative criteria. Both polyclonal CML-specific antibody and CMS-10 significantly reacted with CML-proteins but not with CEL-proteins. It is likely therefore that these antibodies can recognize the difference of one methyl group between CML and CEL. Moreover, CMS-10 significantly reacted with BSA modified with several aldehydes and its reactivity was highly correlated with the CML content, which was determined by high performance liquid chromatography, whereas 6D12 showed a low correlation. These results indicate that CMS-10 can be used to determine the CML contents of modified proteins in a more specific way.

Key words: advanced glycation end products (AGEs), anti-CML antibody, N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(carboxyethyl)lysine (CEL), diabetic complications.

Abbreviations**:** CML, N^{ε}-(carboxymethyl)lysine; CEL, N^{ε}-(carboxyethyl)lysine; AGE(s), advanced glycation end products; BSA, bovine serum albumin; AGE-BSA, glucose-modified AGE-BSA; CPC, conventional polyclonal anti-CML antibody; ELISA, enzyme-linked immunosorbent assay; GO, glyoxal; GA, glycolaldehyde; MG, methylglyoxal; 3-DG, 3-deoxyglucosone; PBS, phosphate-buffered saline.

Proteins exposed to reducing sugars such as glucose undergo nonenzymatic glycation, leading to the progressive formation of advanced glycation end products termed AGE. AGE show such physicochemical properties as fluorescence, a brown color and intra- or inter-molecular cross-linking. Monnier *et al.* (*1*) demonstrated that fluorescent pigments, which show similar fluorescence spectra to AGE, accumulate in the human dura mater in an age-dependent manner and increase during in the pathogenesis of diabetes, suggesting that AGE formation progresses *in vivo*. However, measurement of AGE by means of fluorometric analysis is limited due to its low-specificity. To investigate the histological localization of AGE *in vivo*, we previously prepared a monoclonal antibody against AGE called 6D12, by immunization with AGE-modified bovine serum albumin (AGE-BSA), followed by successive screening for AGE-BSA–positive but BSA-negative clones (*2*). Our subsequent study demonstrated that 6D12 recognizes CML and CML-proteins adducts (*3*). CML is generated through several pathways, such as oxidative cleavage of the Amadori product by the hydroxyl radical (*4*) and peroxynitrite (*5*). In addition to these pathways, CML formation also takes place through glyoxal, which is generated through autoxidation of glucose (*6*), oxidative cleavage of Schiff bases (*7*), and unsaturated fatty acids (*8*). Since CML formation requires oxidation in all pathways, CML is thought to be the important biological markers of oxidative stress *in vivo*. Recent immunological studies involving 6D12 demonstrated that CML modification is involved in normal aging (*9*), as well as in the pathogeneses of several age-enhanced diseases such as diabetic nephropathy (*10*), atherosclerosis (*11*), diabetic retinopathy (*12*), hemodialysis-associated amyloidosis (*13*), chronic renal failure (*14*), and Alzheimer's disease (*15*). Furthermore, CML

*To whom correspondence should be addressed. Phone: +81-96-373-5071, Fax: +81-96-364-6940, E-mail: nagai@kaiju.medic.kumamoto-u.ac.jp

accumulates in hippocampal neurons, neurofibrillary tangles, peripheral nerves, atherosclerotic lesions in rat aortas, atherosclerotic coronary arteries, murine amyloid, the peritoneum in patients on continuous ambulatory peritoneal dialysis (*16*), human skin elastin in actinic elastosis (*17*), cardiac tissues of renal transplant patients (*18*), and pulmonary fibrosis (*19*).

Recent studies demonstrated that methylglyoxal (MG) is generated through the Embden-Meyerhof and polyol pathways, and rapidly reacts with proteins to form MG-derived AGE such as CEL. CEL is detected in human lens proteins at a concentration similar to that of CML, and its accumulation increases with age in parallel with that of CML (*20*). Degenhardt *et al.* (*21*) have also demonstrated that CEL increases in lens proteins and skin collagen with age. These reports clearly demonstrate that CEL is an important AGE that is derived through metabolic pathways.

The purpose of the present study is to determine whether 6D12 and conventionally prepared polyclonal anti-CML antibody cross-react with CEL because of its structural similarity to CML. The results demonstrate that these antibodies significantly reacted not only with CML but also with CEL. These results strongly suggest that some of the CML detected by 6D12 and conventional polyclonal anti-CML antibody (CPC) might have been CEL, and that CML specific antibody could be an important tool for demonstrating the pathological significance of CML.

MATERIALS AND METHODS

Chemicals—β-D-Glucose and MG were purchased from Sigma (St. Louis, MO). Upon analysis with the Hantzsch reaction, the amount of formaldehyde in the MG solution was found to be less than the minimum detectable level. Fatty acid-free bovine serum albumin (BSA), glyoxylic acid and sodium pyruvate were purchased from Wako (Osaka, Japan). Tissue culture medium was from Gibco. All other chemicals were of the best grade available from commercial sources.

Modification of BSA with Aldehydes—Aldehyde-modified BSA was prepared as described in previously (*22*). Briefly, 2 mg/ml of BSA (1.5 mM lysine residues) was incubated at 37°C for 7 days with 33 mM MG, glyoxal (GO), and glycolaldehyde (GA). 3-Deoxyglucosone (3-DG)–modified BSA was prepared by incubating 200 mM 3-DG with 2 mg/ml of BSA at 37°C for 7 days (*23*). Glucose-modified AGE-BSA (AGE-BSA) was prepared under sterile conditions by incubation of 50 mg/ml BSA with 1 M glucose at 37°C for up to 40 weeks, and aliquots were removed from the reaction mixture on days 5, 13, 20, 27, 35, 42, 52 and 40 weeks and dialyzed against phosphate-buffered saline (PBS) (pH 7.4).

Preparation of CML-Modified BSA (CML-BSA) and CEL-Modified BSA (CEL-BSA)—CML-BSA with different CML contents was prepared as described previously (*3*). Briefly, BSA (50 mg/ml) was incubated at 37°C for 24 h with 0.4–100 mM glyoxylic acid and 100 mM sodium cyanoborohydride ($NaBH_3CN$) in 1 ml of 0.2 M sodium phosphate buffer (pH 7.8), followed by dialysis against PBS. CML-KLH was prepared by incubating KLH (5 mg/ml) with 50 mM glyoxylic acid and 100 mM $NaBH_3CN$ at 37°C for 24 h in 1 ml of 0.2 M sodium phosphate buffer, followed by dialysis against PBS. CEL-BSA and CEL-RNase were prepared by incubating RNase (40 mg/ml) with 90 mM pyruvate at room temperature for 12 h in the presence of 5 mM $NaBH_3CN$ in 1 ml of 0.2 M sodium phosphate buffer (pH 7.4), followed by dialysis against PBS (*20*). Hippuryl-CML (*3*) and hippuryl-CEL (*24*) were prepared as described previously.

Preparation of Polyclonal Anti-CML Antibody—The experimental protocol was approved by the local ethics review committee for animal experimentation. To prepare a polyclonal antibody against CML, 1.0 mg of CML-KLH in 50% Freund's complete adjuvant was injected intradermally into 10 skin sites on a rabbit, followed by four booster injections of 0.5 mg of CML-KLH in 50% Freund's incomplete adjuvant. Serum obtained 10 days after the final immunization was subjected to further affinity purification. Formyl-cellulofine gel was coupled to CML-BSA as described previously (*3*). The anti-CML-KLH antiserum (5 ml) was passed over a column (1.2 × 9.0 cm) of formyl-cellulofine-CML-BSA gel. The fraction adsorbed to CML-BSA was eluted with 0.1 M citric acid buffer (pH 3.0). The pooled antibody fraction was neutralized, dialyzed against PBS, and used as CPC. CPC was further applied to Formyl-cellulofine-CEL-BSA gel to obtain an antibody specific for CML.

Preparation of Monoclonal Antibody Specific for CML—Splenic lymphocytes obtained from CML-KLH–immunized BALB/c mice were fused to myeloma P3U1 cells in the presence of polyethylene glycol as described previously (*25*). The hybrid cells were screened, and one cell line, named CMS-10, positive for CML-BSA but negative for CEL-BSA was selected through successive subcloning. This cell line was inoculated into the peritoneal cavities of Balb/c mice and antibody was purified from ascitic fluid by protein G affinity chromatography to IgG_1. CMS-10 thus prepared was used for the ELISA studies described below.

Enzyme-Linked Immunosorbent Assay (ELISA)—For non-competitive ELISA (*3*), each well of a 96-well microtiter plate was coated with 100 μl of the indicated concentration of sample in PBS, followed by incubation for 2 h. The wells were washed three times with PBS containing 0.05% Tween 20 (buffer A). The wells were then blocked with 0.5% gelatin in PBS for 1 h. After triplicate washing with buffer A, the wells were incubated for 1 h with 100 μl of the indicated concentration of antibodies. After triplicate washing with buffer A, the wells were incubated with horseradish peroxidase (HRP)–conjugated anti-mouse IgG antibody (Kirkegaard & Perry Laboratories, Maryland, USA), followed by reaction with 1,2-phenylenediamine dihydrochloride. The reaction was terminated with 100 μl of 1.0 M sulfuric acid, and the absorbance was read at 492 nm with a micro-ELISA plate reader. For competitive ELISA (*3*), each well of a 96-well microtiter plate was coated 2 h with 100 μl of the indicated concentration of antigen in PBS. The wells were washed three times with buffer A and then blocked for 1 h with 0.5% gelatin in PBS. After triplicate washing with buffer A, 50 μl of the sample to be tested and the same volume of antibody was added to each well, followed by 1 h-incubation. The antibodies bound to the wells were detected with HRP-conjugated anti-mouse or anti-rabbit IgG antibod-

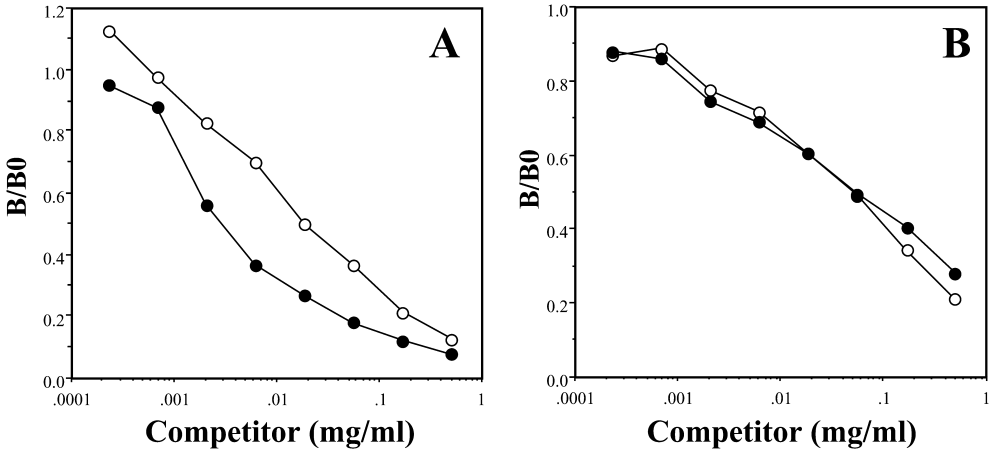

Fig. 1. **Immunoreactivity of 6D12 and CPC with CML- and CEL-proteins.** Immunoreactivity was assayed by competitive ELISA. Each well was coated with 100 μl of 1 μg/ml CML-BSA and then blocked with 0.5% gelatin as described under "MATERIALS AND METHODS." Fifty microliters of CML-BSA (open circles) (12.9 mol CML/mol BSA) or CEL-BSA (closed circles) (8.9 mol CEL/mol BSA) and the same volume of 0.1 μg/ml 6D12 (A) or 1 μg/ml CPC (B) were added to each well. The wells were then washed with buffer A, and the antibodies bound to wells were detected with HRP-conjugated anti-mouse IgG (A) or anti-rabbit IgG (B).

ies in a manner similar to that described above for non-competitive ELISA.

Determination of CML Contents by HPLC—The CML contents of modified BSA preparations were determined by amino acid analysis after acid hydrolysis with 6 N HCl for 24 h at 110°C with an amino acid analyzer (L-8500A, Hitachi) (Tokyo, Japan) equipped with an ion exchange column (#2622 SC, 4.6 × 80 mm, Hitachi), as described previously (*24*).

Preparation of human Lens Proteins—Human lenses from normal subjects (non-diabetic, non-cataractous) were purchased through the National Disease Research Interchange and were stored at –80°C until use. The lenses were homogenized as described previously (*9*). Briefly, each lens was homogenized in PBS, followed by centrifugation at 10,000 × *g* for 30 min at 4°C, and then the protein concentration of the supernatant was measured with bicinchoninic acid protein assay reagent (Pierce, Rockford, IL).

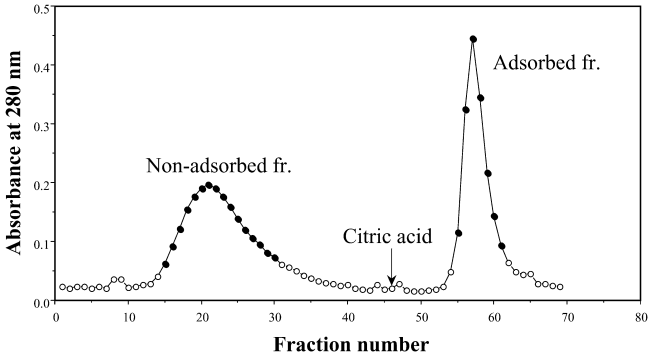

Fig. 2. **Separation of polyclonal CML-specific antibody from CPC by CEL-BSA–conjugated affinity chromatography.** CPC (6.1 mg) purified with CML-BSA–conjugated column was further applied to a CEL-BSA–conjugated column, which was washed with 50 ml of PBS to obtain the non-adsorbed fraction. The adsorbed fraction was eluted with 30 ml of 0.1 M citric acid (pH 3.0) and each fraction (1.0 ml) was monitored for absorbance at 280 nm. Both the non-adsorbed fraction (1.6 mg) and the adsorbed fraction (2.2 mg) were pooled, concentrated and dialyzed against PBS.

RESULTS

Immunoreactivity of 6D12 and CPC with CML- and CEL-Proteins—The immunoreactivities of the monoclonal anti-CML antibody (6D12) and the polyclonal antibody were determined by competitive ELISA. The reactivity of 6D12 with CML-BSA was significantly competed for dose-dependently not only by CML-BSA but also by CEL-BSA, indicating that 6D12 recognized both CML- and CEL-protein adducts (Fig. 1A). These data strongly suggest that the epitope of 6D12 is a structure common to CML and CEL-protein adducts. CPC showed similar reactivity with CML-BSA and CEL-BSA (Fig. 1B), indicating that CPC might include an antibody population recognizing CML-protein adducts specifically, and an antibody population recognizing both CML- and CEL-protein adducts. To test this possibility, CPC was further subjected to CEL-BSA-conjugated affinity chromatography to separate the CML-specific antibody population. Application of CPC to the column resulted in separation of non-adsorbed and adsorbed fractions (Fig. 2). The non-adsorbed fraction reacted positively with CML-RNase, but not CEL-RNase (Fig. 3A), whereas the adsorbed fraction showed significant reactivity with both CML-RNase and CEL-RNase (Fig. 3B). These results clearly indicated that the polyclonal antibody obtained on immunization with CML-KLH included at least two antibody populations, such as one recognizing CML but not CEL, and one recognizing both CML and CEL.

Preparation of Monoclonal Antibody Specific for CML—Since the non-adsorbed fraction obtained with the CEL-BSA–conjugated affinity column contained an antibody recognizing CML exclusively (Fig. 3A), an attempt was made to prepare a monoclonal antibody recognizing CML but not CEL. Spleen lymphocytes immunized with CML-KLH were fused with myeloma cells and then subjected to successive subcloning according to CML-BSA–positive but CEL-BSA–negative criteria. One cell line named CMS-10 was obtained. Non-competitive ELISA showed that CMS-10 significantly reacted with CML-BSA in a dose-dependent manner, whereas its reactivity with CEL-BSA was negligible (Fig. 4A). Consistent results were obtained on competitive ELISA; the reaction of CMS-10 with CML-BSA was significantly competed by

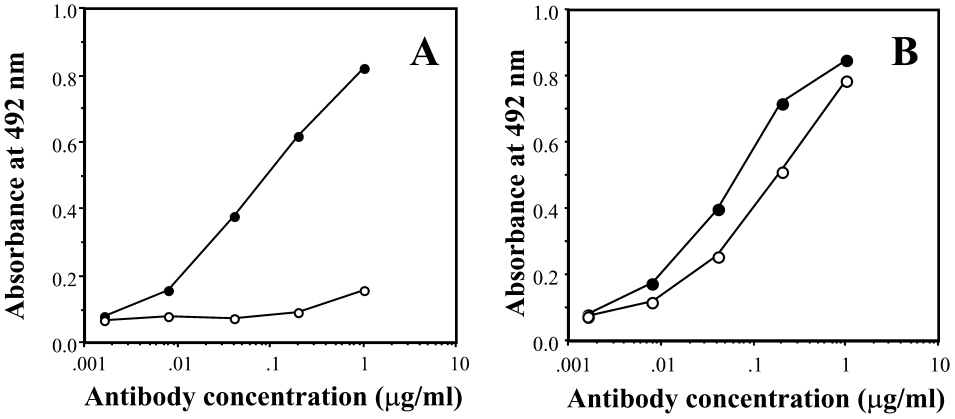

Fig. 3. **Immunoreactivity of the non-adsorbed fraction and adsorbed fractions obtained from a CEL-BSA–conjugated column.** The immunoreactivities of the non-adsorbed and adsorbed fractions with CML- and CEL-RNase were determined by non-competitive ELISA. Each well was coated with 100 μl of 1 μg/ml CML-RNase (closed circles) or CEL-RNase (open circles), and then blocked with 0.5% gelatin. After triplicate washing with buffer A, the wells were incubated for 1 h with 100 μl of the indicated concentration of non-adsorbed fraction (A) or adsorbed fraction (B). The wells were washed with buffer A, and then the antibodies bound to the wells were detected with HRP-conjugated anti-rabbit IgG.

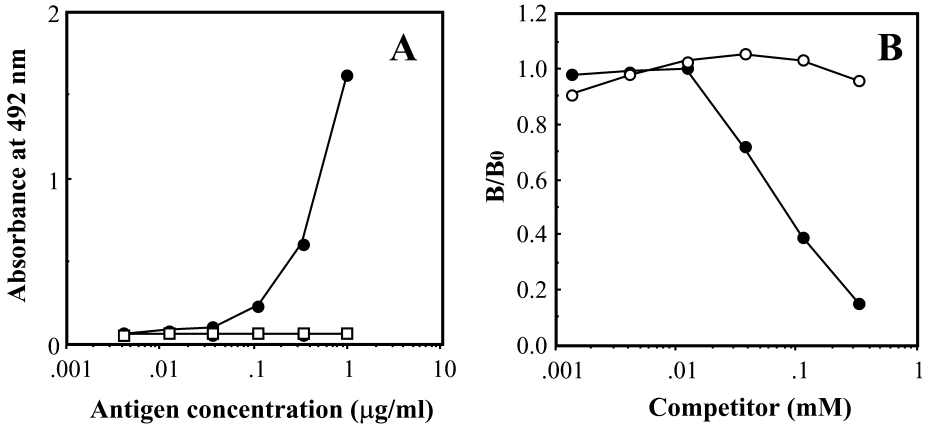

Fig. 4. **Reactivity of monoclonal antibody specific for CML.** The immunoreactivity of CMS-10 was determined by non-competitive and competitive ELISA. (A) Non-competitive ELISA: Each well was coated with 100 μl of the indicated concentration of CML-BSA (closed circles) or CEL-BSA (open squares), and then blocked with 0.5% gelatin. After triplicate washing with buffer A, the wells were incubated for 1 h with 100 μl of 1 μg/ml of CMS-10. (B) Competitive ELISA: Each well was coated with 100 μl of 0.5 μg/ml CML-BSA and then blocked with 0.5% gelatin. Fifty microliters of hippuryl-CML (closed circles) or hippuryl-CEL (open circles) and the same volume of 1 μg/ml CMS-10 were added to each well. The wells were then washed with buffer A, and then the antibodies bound to wells were detected with HRP-conjugated anti-mouse IgG.

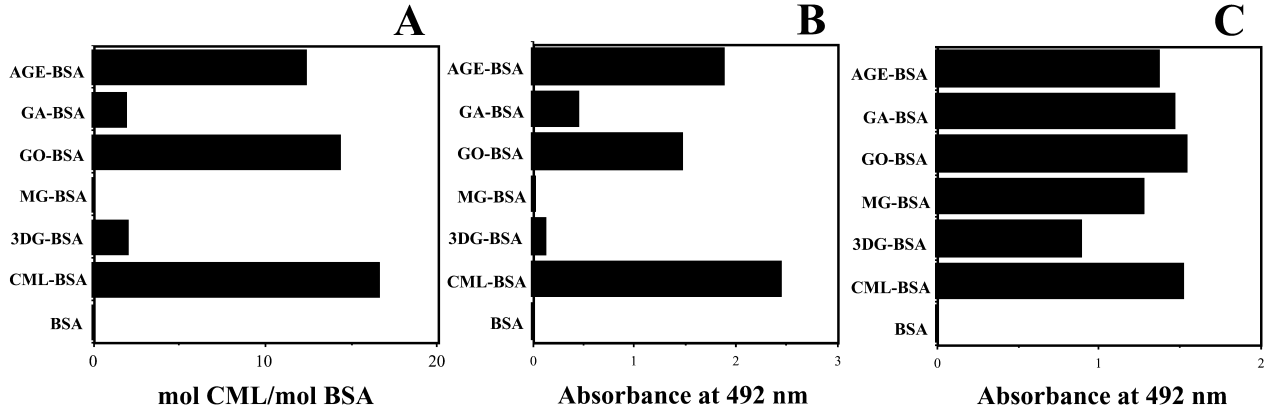

Fig. 5. **Correlation of CML contents of aldehyde-modified proteins and their immunoreactivities.** (A) The CML content of each aldehyde-modified BSA was determined by HPLC after acid hydrolysis and was expressed mol/mol of BSA. The reactivity of aldehyde-modified BSA with CMS-10 (B) and 6D12 (C) was determined by non-competitive ELISA. Each well was coated with 100 μl of 1 μg/ml each antigen, and 1 μg/ml CMS-10 (B) and 0.1 μg/ml 6D12 (C) were used as primary antibodies.

hippuryl-CML, but not by hippuryl-CEL (Fig. 4B). It is likely therefore that CMS-10 can recognize the difference between CEL and CML.

Correlation of CML Contents of Aldehyde-Modified Proteins and Their Immunoreactivities—Recent studies have clarified that several intermediate aldehydes, such as GA, GO, MG and 3-DG, are involved in the formation of AGE-structures (*22*, *23*). BSA was modified with these aldehydes and the CML content of each modified-protein was determined by HPLC after hydrolysis. The CML content of GO-modified BSA (14.5 mol CML/mol BSA) was similar to those of AGE-BSA (12.5 mol CML/mol BSA)

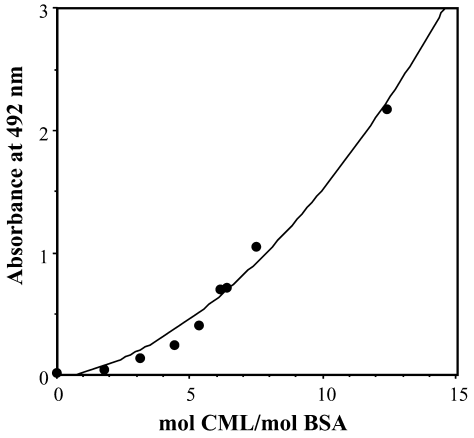

Fig. 6. **Correlation of CML contents of AGE-BSA samples with their immunoreactivities.** The reactivity of CMS-10 with AGE-BSA preparations with different incubation periods was determined by non-competitive ELISA in the same way as in Fig. 5. Each well was coated with 100 μl of 1 μg/ml each AGE-BSA, and 1 μg/ml CMS-10 was used as primary antibodies. Immunoreactivity was plotted against CML-content.

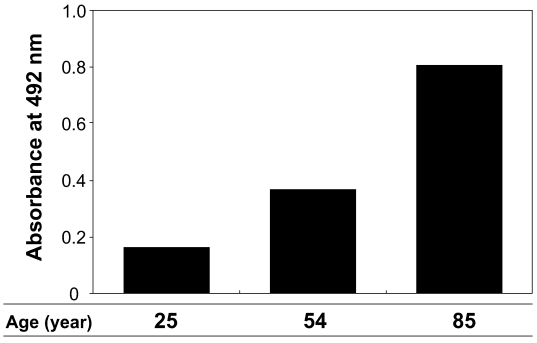

Fig. 7. **Immunochemical reactivity of CMS-10 with human lens protein.** Each well was coated with human lens proteins (10 μg/ml) and then blocked with 0.5% gelatin, followed by triplicate washing with buffer A. Each well was incubated for 1 h with 100 μl of 10 μg/ml of CMS-10. The amount of the antibody bound to each well was determined with HRP-conjugated anti-mouse IgG.

and CML-BSA (16.7 mol CML/mol BSA), but higher than those of GA-BSA (2.1 mol CML/mol BSA) and 3-DG-BSA (2.0 mol CML/mol BSA) (Fig. 5A). The CML content of MG-modified BSA was less than detectable level (Fig. 5A). The immunoreactivities of these aldehyde-modified BSAs with CMS-10 were measured by non-competitive ELISA (Fig. 5B). The extents of their immunoreactivity with CMS-10 were closely similar to their CML contents (Fig. 5A), indicating that the reactivity with CMS-10 reflected their CML contents. However, when we measured the immunoreactivities of these modified proteins with 6D12, a monoclonal antibody recognizing both CML and CEL, the immunoreactivities of GA-modified BSA, 3-DG–modified BSA and MG-modified BSA were significantly as high as that of GO-modified BSA (Fig. 5C). The extents of the immunoreactivity of 6D12 (Fig. 5C) did not show any similarity to those of the immunoreactivity of CMS-10 (Fig. 5B) or the CML contents of these modified proteins (Fig. 5A), suggesting that CML quantification with CMS-10 should be much more accurate than that with 6D12.

Correlation of CML Contents of AGE-Modified Proteins with Their Immunoreactivities with CMS-10—To determine the correlation between the CML contents of the AGE-BSAs and their immunoreactivities with CMS-10, nine AGE-BSA samples with different CML contents were prepared. The immunoreactivities of CMS-10 with these samples were plotted against CML-content. The immunoreactivity gradually increased up to 4.5 mol CML/mol BSA. Upon a further increase in CML content, however, it increased proportionally with the CML content of BSA (Fig. 6). In contrast, the immunoreactivity of 6D12 with these AGE-BSA samples increased rapidly up to approximately 5 mol CML/mol BSA and then appeared to reach a plateau (data not shown).

The Immunochemical Reactivity of CMS-10 with Human Lens Proteins—We also used CMS-10 to measure the CML contents of human lens proteins. CMS-10 significantly recognized lens proteins and its reactivity increased steadily with lens age (Fig. 7), indicating that this antibody could detect CML in biological materials.

DISCUSSION

AGE research has enormously been expanded to the medical field since the 1990s because of the development of polyclonal and monoclonal antibodies against AGE-modified proteins. Our immunochemical approaches involving 6D12 have greatly contributed to understanding of the biological significance of AGE in the pathogeneses of age-enhanced diseases (*9–19*, *26*). Our subsequent study demonstrated that 6D12 recognizes CML and CML-protein adducts (*3*). In this connection, our group (*3*) and Reddy *et al.* (*27*) also demonstrated that CML is a major antigenic AGE structure. Schmidt *et al.* demonstrated that CML is recognized as a ligand structure by a cell surface receptor for AGE (RAGE), and the CML-RAGE interaction activates the cell signaling pathway *via* NF-κB and enhances the expression of vascular cell adhesion molecule-1 (VCAM-1) in human umbilical vein endothelial cells (*28*). Therefore, CML is now widely accepted as one of the most important structures generated through post-translational modifications that contribute to the pathogeneses of age-related disorders such as diabetic complications and atherosclerosis.

MG rapidly reacts with proteins to form MG-derived AGEs such as CEL (*20*, *21*), imidazolone (*29*), argpyrimidine (*30*), and imidazolium salt cross-link like methylglyoxal-lysine dimer (MOLD) (*31*) *in vivo*. Oya *et al.* (*30*) prepared a monoclonal antibody, named mAb6B, against MG-modified proteins and identified its epitope as argpyrimidine. Immunohistochemical analysis with mAb6B revealed the localization of argpyrimidine in the intima and media of small arteries in renal tissues from diabetic patients. Nagaraj *et al.* (*32*) quantified MOLD in human plasma proteins by reverse phase high performance liquid chromatography (RP-HPLC), and showed that cross-linking of plasma proteins by MOLD was increased in diabetes. The amounts of MOLD in serum proteins from non-diabetic uremic patients determined by electrospray ionization/mass spectrometry/mass spectrometry (ESI/MS/MS) were about 3-fold higher than those in age-

matched controls (*33*). We previously measured CEL, one of the MG-derived AGE structures, by HPLC to investigate the involvement of MG in AGE formation. However, detection of CEL in AGE-proteins with our HPLC system was unsuccessful due to co-elution of CEL with CML. This co-elution is because of the similar chemical structures of CEL and CML, which makes separation of these compounds difficult (*3*). We, therefore, developed a new HPLC system in which CEL and CML are measured simultaneously in a single HPLC run and found that glucose-derived AGE-proteins contain not only CML but also CEL (*24*), indicating that MG plays a role as a reactive intermediate for AGE formation during the incubation of a protein with glucose. Based on our analytical experience, we examined the possibility that anti-CML antibody might recognize the CEL as well as CML due to their structural similarity.

In the present study, we demonstrated that CPC and 6D12 recognize not only CML but also CEL. Furthermore, since CPC was separated into two fractions, non-adsorbed and adsorbed ones, on CEL-BSA–conjugated affinity chromatography, and the non-adsorbed fraction showed high specificity for CML (Fig. 2), we also prepared a monoclonal CML-specific antibody named CMS-10. The reactivity of CMS-10 with aldehyde-modified BSAs was highly correlated with their CML contents, which were determined by HPLC (Fig. 5B), whereas 6D12 showed significant reactivity with all of the modified-BSA preparations without any correlation with their CML contents (Fig. 5C), indicating that determination of the CML content with CMS-10 is much more accurate than that with 6D12 and CPC.

We previously prepared polyclonal mono-specific anti-CML antibody by immunization with CML-BSA, followed by purification by CML-BSA–conjugated affinity chromatography (*34*). This antibody significantly reacted with thickened vascular walls in diabetic retinas. Degenhardt *et al.* demonstrated that CML is the major epitope of polyclonal anti-AGE antibody obtained by immunization of rabbit with AGE-RNase, and that the CML contents in the sera of continuous ambulatory peritoneal dialysis and hemodialysis patients are higher than those in control subjects. Schleicher *et al.* (*35*) also originally prepared polyclonal anti-CML antibody by immunization of rabbit with CML-KLH, followed by purification by CML-BSA–conjugated affinity chromatography. This antibody significantly reacted with atherosclerotic plaques and foamed macrophages. Kato *et al.* prepared polyclonal anti-CML antibody by immunizing rabbit with CML-KLH and detected CML in copper-oxidized low density lipoprotein (*36*). Hamelin *et al.* also prepared polyclonal anti-CML antibody by immunization with CML-BSA and demonstrated that the CML level in the serum of a 27-month-old rat was 50% higher than in that of a 3-month-old rat (*37*). However, since they did not measure the cross-reaction of their antibodies with CEL, some of the CML detected with their antibodies might have been CEL.

As described in the introduction, CML is formed through several pathways such as oxidative cleavage of Amadori products (*4*, *5*), Schiff base (*7*), autoxidation of glucose (*6*), and unsaturated fatty acids (*8*) *via* glyoxal. Since CML formation is inhibited by antioxidants and antioxidative conditions (*38*), reactive oxygen species are believed to be involved in CML formation. In contrast, most MG, a precursor for CEL formation, is generated through a metabolic pathway through the degradation of triosephosphate, and CEL formation progresses independent of oxidation chemistry, demonstrating that the pathway for the formation of CML is obviously different from that for CEL. Therefore, specific detection of CML and CEL is fundamental for determining which pathways are involved in the pathogeneses of diseases and for developing therapeutic strategies.

In conclusion, our study clearly demonstrated that a conventionally prepared anti-CML antibody recognizes not only CML but also CEL. We also used CMS-10 to measure the CML contents of human lens proteins. The antibody significantly reacted with lens proteins obtained from a 25-year-old and the reactivity increased in an age-dependent manner (Fig. 7), suggesting that immunochemical detection involving CML-specific antibody is an excellent tool for determining the CML content. Further studies will be important to demonstrate the localization of CML in pathological tissues with specific antibodies.

We are grateful to Kaori Takaki, Yuka Unno, Yu-ichiro Sakamoto, Masaharu Hori and Maki Satoh of our laboratory for their collaborative endeavors. This work was supported in part by Grants-in-Aid for Scientific Research (13470228 to SH and 16790511 to RN) from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES

1. Monnier, V.M., Kohn, R.R., and Cerami, A. (1984) Accelerated age-related browning of human collagen in diabetes mellitus. *Proc. Natl Acad. Sci. USA* **81**, 583–587
2. Horiuchi, S., Araki, N., and Morino, Y. (1991) Immunochemical approach to characterize advanced glycation end products of the Maillard reaction. Evidence for the presence of a common structure. *J. Biol. Chem.* **266**, 7329–7332
3. Ikeda, K., Higashi, T., Sano, H., Jinnouchi, Y., Yoshida, M., Araki, T., Ueda, S., and Horiuchi, S. (1996) N^{ε}-(carboxymethyl)lysine protein adduct is a major immunological epitope in proteins modified with advanced glycation end products of the Maillard reaction. *Biochemistry* **35**, 8075–8083
4. Nagai, R., Ikeda, K., Higashi, T., Sano, H., Jinnouchi, Y., Araki, T., and Horiuchi, S. (1997) Hydroxyl radical mediates N^{ε}-(carboxymethyl)lysine formation from Amadori product. *Biochem. Biophys. Res. Commun.* **234**, 167–172
5. Nagai, R., Unno, Y., Hayashi, M.C., Masuda, S., Hayase, F., Kinae, , N., and Horiuchi, S. (2002) Peroxynitrite induces formation of N^{ε}-(carboxymethyl) lysine by the cleavage of Amadori product and generation of glucosone and glyoxal from glucose: novel pathways for protein modification by peroxynitrite. *Diabetes* **51**, 2833–2889
6. Wells-Knecht, K.J., Zyzak, D.V., Litchfield, J.E., Thorpe, S.R., and Baynes, J.W. (1995) Mechanism of autoxidative glycosylation: identification of glyoxal and arabinose as intermediates in the autoxidative modification of proteins by glucose. *Biochemistry* **34**, 3702–3709
7. Glomb, M.A. and Monnier, V.M. (1995) Mechanism of protein modification by glyoxal and glycolaldehyde, reactive intermediates of the Maillard reaction. *J. Biol. Chem.* **270**, 10017–10026
8. Fu, M.X., Requena, J.R., Jenkins, A.J., Lyons, T.J., Baynes, J.W., and Thorpe, S.R. (1996) The advanced glycation end product, N^{ε}-(carboxymethyl)lysine, is a product of both lipid peroxidation and glycoxidation reactions. *J. Biol. Chem.* **271**, 9982–9986

9. Araki, N., Ueno, N., Chakrabarti, B., Morino, Y., and Horiuchi, S. (1992) Immunochemical evidence for the presence of advanced glycation end products in human lens proteins and its positive correlation with aging. *J. Biol. Chem.* **267**, 10211–10214
10. Imai, N., Nishi, S., Suzuki, Y., Karasawa, R., Ueno, M., Shimada, H., Kawashima, S., Nakamaru, T., Miyakawa, Y., Araki, N., Horiuchi, S., Gejyo, F., and Arakawa, M. (1997) Histological localization of advanced glycosylation end products in the progression of diabetic nephropathy. *Nephron* **76**, 153–160
11. Kume, S., Takeya, M., Mori, T., Araki, N., Suzuki, H., Horiuchi, S., Kodama, T., Miyauchi, Y., and Takahashi, K. (1995) Immunohistochemical and ultrastructural detection of advanced glycation end products in atherosclerotic lesions of human aorta with a novel specific monoclonal antibody. *Amer. J. Pathol.* **147**, 654–667
12. Hammes, H.P., Weiss, A., Hess, S., Araki, N., Horiuchi, S., Brownlee, M., and Preissner, K.T. (1996) Modification of vitronectin by advanced glycation alters functional properties *in vitro* and in the diabetic retina. *Lab. Invest.* **75**, 325–338
13. Miyata, T., Oda, O., Inagi, R., Iida, Y., Araki, N., Yamada, N., Horiuchi. S., Taniguchi, N., Maeda, K., and Kinoshita, T. (1993) beta 2-Microglobulin modified with advanced glycation end products is a major component of hemodialysis-associated amyloidosis. *J. Clin. Invest.* **92**, 1243–1252
14. Yamada, K., Miyahara, Y., Hamaguchi, K., Nakayama, M., Nakano, H., Nozaki, O., Miura, Y., Suzuki, S., Tuchida, H., Mimura, N., *et al.* (1994) Immunohistochemical study of human advanced glycosylation end-products (AGE) in chronic renal failure. *Clin. Nephrol.* **42**, 354–361
15. Smith, M.A., Taneda, S., Richey, P.L., Miyata, S., Yan, S.D., Stern, D., Sayre, L.M., Monnier, V.M., and Perry, G. (1995) Advanced Maillard reaction end products are associated with Alzheimer disease pathology. *Proc. Natl Acad. Sci. USA* **91**, 5710–5714
16. Nakayama, M., Kawaguchi, Y., Yamada, K., Hasegawa, T., Takazoe, K., Katoh, N., Hayakawa, H., Osaka, N., Yamamoto, H., Ogawa, A., Kubo, H., Shigematsu, T., Sakai, O., and Horiuchi, S. (1997) Immunohistochemical detection of advanced glycosylation end-products in the peritoneum and its possible pathophysiological role in CAPD. *Kidney Int.* **51**, 182–186
17. Mizutari, K., Ono, T., Ikeda, K., Kayashima, K., and Horiuchi, S. (1997) Photo-enhanced modification of human skin elastin in actinic elastosis by N^{ε}-(carboxymethyl)lysine, one of the glycoxidation products of the Maillard reaction. *J. Invest. Dermatol.* **108**, 797–802
18. Yoshida, S., Yamada, K., Hamaguchi, K., Nishimura, M., Hatakeyama, E., Tsuchida, H., Sakamoto, K., Kashiwabara, H., Yokoyama, T., Ikeda, K., and Horiuchi, S. (1998) Immunohistochemical study of human advanced glycation end-products (AGE) and growth factors in cardiac tissues of patients on maintenance dialysis and with kidney transplantation. *Clin. Nephrol.* **49**, 273–280
19. Matsuse, T., Ohga, E., Teramoto, S., Fukayama, M., Nagai, R., Horiuchi, S., and Ouchi, Y. (1998) Immunohistochemical localisation of advanced glycation end products in pulmonary fibrosis. *J. Clin. Pathol.* **51**, 515–519
20. Ahmed, M.U., Frye, E.B., Degenhardt, T.P., Thorpe, S.R., and Baynes, J.W. (1997) N^{ε}-(carboxyethyl)lysine, a product of the chemical modification of proteins by methylglyoxal, increases with age in human lens proteins. *Biochem. J.* **324**, 565–570
21. Degenhardt, T.P., Thorpe, S.R., and Baynes, J.W. (1998) Chemical modification of proteins by methylglyoxal. *Cell. Mol. Biol.* **44**, 1139–1145
22. Nagai, R., Matsumoto, K., Ling, X., Suzuki, H., Araki, T., and Horiuchi, S. (2000) Glycolaldehyde, a reactive intermediate for advanced glycation end products, plays an important role in the generation of an active ligand for the macrophage scavenger receptor. *Diabetes* **49**, 1714–1723
23. Jono, T., Nagai, R., Ling, X., Ahmed, N., Thornalley, P.J. Takeya, M., and Horiuchi, S. (2004) N^{ε}-(carboxymethyl)lysine and 3-DG-imidazolone are major AGE structures in protein modification by 3-deoxyglucosone. *J. Biochem.* **136**, 351–358
24. Nagai, R., Araki, T., Hayashi, C.M., Hayase, F., and Horiuchi, S. (2003) Identification of N^{ε}-(carboxyethyl)lysine, one of the methylglyoxal-derived AGE structures, in glucose-modified protein: mechanism for protein modification by reactive aldehydes. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **788**, 75–84
25. Nagai, R., Hayashi, C.M., Xia, L., Takeya, M., and Horiuchi, S. (2002) Identification in human atherosclerotic lesions of GA-pyridine, a novel structure derived from glycolaldehyde-modified proteins. *J. Biol. Chem.* **277**, 48905–48912
26. Nagai, R. and Horiuchi, S. (2003) Application of monoclonal antibody libraries for the measurement of glycation adducts. *Biochem. Soc. Trans* **31**, 1438–1440
27. Reddy, S., Bichler, J., Wells-Knecht, K.J., Thorpe, S.R., and Baynes, J.W. (1995) N^{ε}-(carboxymethyl)lysine is a dominant advanced glycation end product (AGE) antigen in tissue proteins. *Biochemistry* **34**, 10872–10878
28. Kislinger, T., Fu, C., Huber, B., Qu, W., Taguchi, A., Du Yan S, Hofmann, M., Yan, S.F., Pischetsrieder, M., Stern, D., and Schmidt, A.M. (1999) N^{ε}-(carboxymethyl)lysine adducts of proteins are ligands for receptor for advanced glycation end products that activate cell signaling pathways and modulate gene expression. *J. Biol. Chem.* **274**, 31740–31749
29. Westwood, M.E., Argirov, O.K., Abordo, E.A., and Thornalley, P.J. (1997) Methylglyoxal-modified arginine residues—a signal for receptor-mediated endocytosis and degradation of proteins by monocytic THP-1 cells. *Biochim. Biophys. Acta* **1356**, 84–94
30. Oya, T., Hattori, N., Mizuno, Y., Miyata, S., Maeda, S., Osawa, T., and Uchida, K. (1999) Methylglyoxal modification of protein. Chemical and immunochemical characterization of methylglyoxal-arginine adducts. *J. Biol. Chem.* **274**, 18492–18502
31. Frye, E.B., Degenhardt, T.P., Thorpe, S.R., and Baynes, J.W. (1998) Role of the Maillard reaction in aging of tissue proteins. Advanced glycation end product-dependent increase in imidazolium cross-links in human lens proteins. *J. Biol. Chem.* **273**, 18714–18719
32. Nagaraj, R.H., Shipanova, I.N., and Faust, F.M. (1996) Protein cross-linking by the Maillard reaction. Isolation, characterization, and *in vivo* detection of a lysine-lysine cross-link derived from methylglyoxal. *J. Biol. Chem.* **271**, 19338–19345
33. Odani, H., Shinzato, T., Usami, J., Matsumoto, Y., Frye, E.B., Baynes, J.W., and Maeda, K. (1998) Imidazolium crosslinks derived from reaction of lysine with glyoxal and methylglyoxal are increased in serum proteins of uremic patients: evidence for increased oxidative stress in uremia. *FEBS Lett.* **427**, 381–385
34. Murata, T., Nagai, R., Ishibashi, T., Inomuta, H., Ikeda, K., and Horiuchi, S. (1997) The relationship between accumulation of advanced glycation end products and expression of vascular endothelial growth factor in human diabetic retinas. *Diabetologia* **40**, 764–769
35. Schleicher, E.D., Wagner, E., and Nerlich, A.G. (1997) Increased accumulation of the glycoxidation product N^{ε}-(carboxymethyl)lysine in human tissues in diabetes and aging. *J. Clin. Invest.* **99**, 457–468
36. Kato, Y., Tokunaga, K., and Osawa, T. (1996) Immunochemical detection of carboxymethylated Apo B-100 in copper-oxidized LDL. *Biochem. Biophys. Res. Commun.* **226**, 923–927
37. Hamelin, M., Borot-Laloi, C., Friguet, B., and Bakala, H. (2003) Increased level of glycoxidation product N^{ε}-(carboxymethyl)lysine in rat serum and urine proteins with aging: link with glycoxidative damage accumulation in kidney. *Arch. Biochem. Biophys.* **411**, 215–222
38. Fu, M.X., Wells-Knecht, K.J., Blackledge, J.A., Lyons, T.J., Thorpe, S.R., and Baynes, J.W. (1994) Glycation, glycoxidation, and cross-linking of collagen by glucose. Kinetics, mechanisms, and inhibition of late stages of the Maillard reaction. *Diabetes* **43**, 676–683