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Immunological Evidence that Non-carboxymethyllysine Advanced Glycation End-products Are Produced from Short Chain Sugars and Dicarbonyl Compounds in vivo

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Abstract

Background: The Maillard reaction that leads to the formation of advanced glycation end-products (AGE) plays an important role in the pathogenesis of angiopathy in diabetic patients and in the aging process. Recently, it was proposed that AGE were not only created by glucose, but also by dicarbonyl compounds derived from the Maillard reaction, autoxidation of sugars and other metabolic pathways of glucose. In this study, we developed four types of non- carboxymethyllysine (CML) anti-AGE antibodies that recognized proteins modified by incubation with short chain sugars and dicarbonyl compounds.
Materials and Methods: AGE-modified serum albumins were prepared by incubation of rabbit serum albumin with glyceraldehyde, glycolaldehyde, methylglyoxal or glyoxal. After immunization of rabbits, four types of AGE-specific antisera were obtained that were specific for the AGE modification. To separate non-CML AGE antibodies (Ab) (non-CML AGE-Ab-2, -3, -4, and -5), these anti-AGE antisera were subjected to affinity chromatography on a matrix coupled with four kinds of AGE bovine serum albumin (BSA) or CML-BSA. These non-CML AGE antibodies were used to investigate the AGE content of serum obtained from diabetic patients on hemodialysis.
Results: Characterization of the four types of non-CML AGE antibodies obtained by immunoaffinity chromatography was performed by competitive ELISA and immunoblot analysis. Non-CML AGE-Ab-2 cross-reacted with the protein modified by glyceraldehyde or glycolaldehyde. Non-CML AGE-Ab-3 and -Ab-4 specifically cross-reacted with protein modified by glycolaldehyde and methylglyoxal, respectively. Non-CML AGE-Ab-5 cross-reacted with protein modified with glyoxal as well as methylglyoxal and glycolaldehyde. Three kinds of non-CML AGE (AGE-2, -4, and -5) were detected in diabetic serum as three peaks with apparent molecular weights of 200, 1.15, and 0.85 kD; whereas, AGE-3 was detected as two peaks with apparent molecular weights of 200 and 0.85 kD.
Conclusion: We propose that various types of non-CML AGE are formed by the Maillard reaction, sugar autoxidation and sugar metabolism. These antibodies enable us to identify such compounds created by the Maillard reaction in vivo.

Introduction

The Maillard reaction, a nonenzymatic reaction between ketones or aldehydes and amino groups of macromolecules, contributes to the aging of proteins and to complications associated with diabetes (1–5). In hyperglycemia associated with diabetes, glucose reacts with the free amino groups of amino acids, proteins, phospholipids and nucleic acids. This process begins with the conversion of reversible Schiff base adducts to more stable, covalently bound Amadori rearrangement products. Over the course of weeks

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to months, the Amadori products undergo further rearrangement reactions to form irreversibly bound moieties called advanced glycation end-products (AGE). Many AGE have fluorescent and covalent cross-linking properties.

Recently, some studies have suggested that AGE are produced not only from sugars such as glucose, but also dicarbonyl compounds derived from Maillard reactions, autoxidation of sugars and other metabolic pathways (Scheme 1). In model systems, AGE have been shown to arise from both metalcatalyzed autoxidation of glucose with the dicarbonyl glyoxal and arabinose as intermediates (6), and from the decomposition of Amadori products to the reactive dicarbonyl 3-deoxyglucosone (7). The dicarbonyl methyglyoxal, produced by nonenzymatic fragmentation of triose phosphates (glyceraldehyde-3-phosphate, etc.) of the glycolytic intermediates, also forms AGE in vitro and may be a major source of intracellular and plasma AGE (8).

Methylglyoxal and glyoxal have been shown to modify proteins through the Maillard reaction. Several tissues and the plasma of diabetic individuals exhibit increased levels of methylglyoxal (9–11) and even the normal human lens has relatively high levels (12). Although formation of cross-linking structures by glyoxal and glycolaldehyde occurs in vitro (6,13), there is still no evidence for such cross-linking in vivo.

To better understand the role of short chain sugars (glyceraldehyde and glycolaldehyde) and dicarbonyl compounds (methylglyoxal and glyoxal) in the modification of proteins in diabetes, we developed non-carboxymethyllysine (CML) anti-AGE antibodies that recognize serum proteins modified by these compounds. These antibodies have enabled us to better identify the compounds involved in the Maillard reaction in vivo.

Materials and Methods

Materials

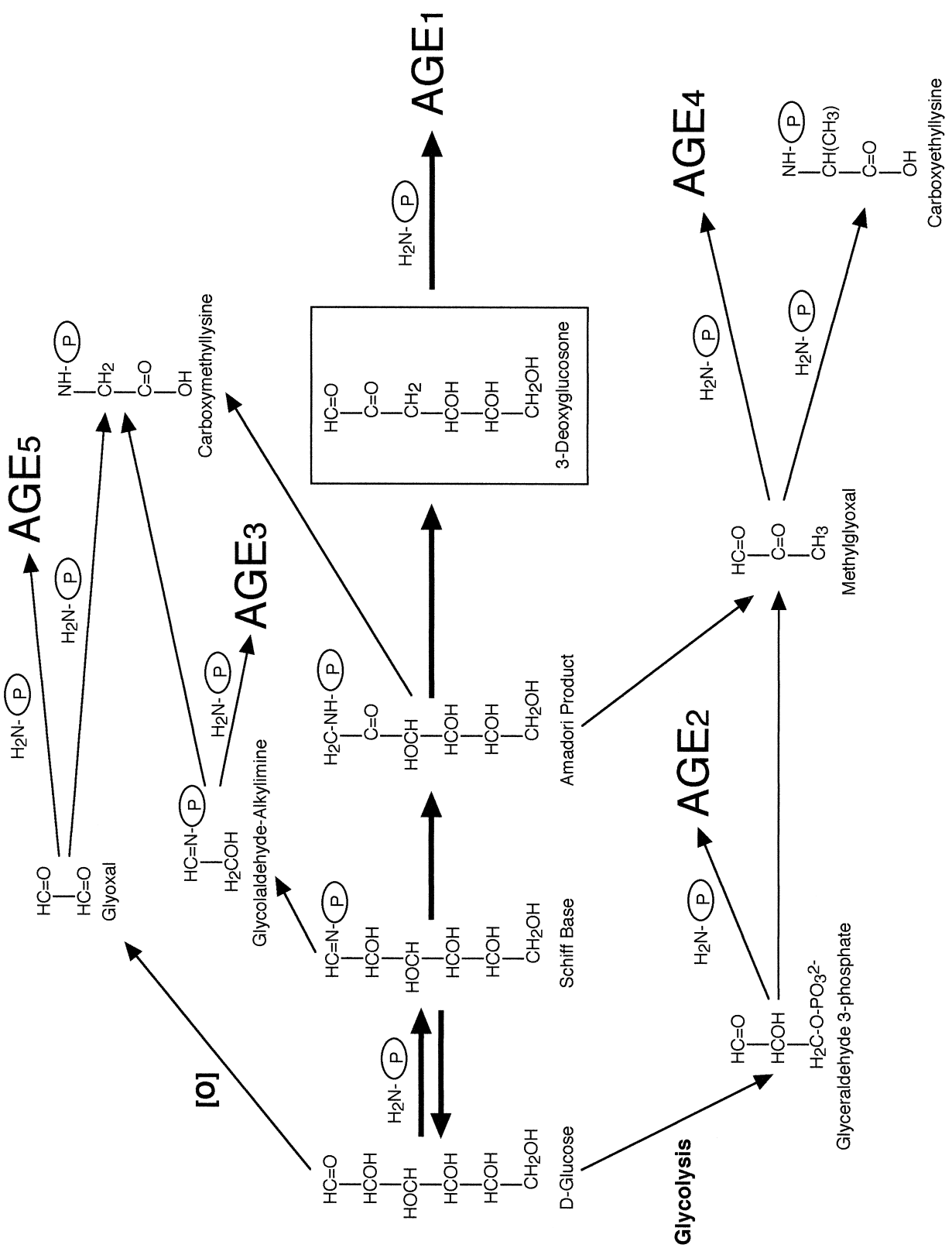
Bovine serum albumin (BSA), rabbit serum albumin (RSA), and methylglyoxal were purchased from Sigma Chemical Co. (St. Louis, MO). D-glyceraldehyde and glycolaldehyde were purchased from Nacalai Tesque (Kyoto, Japan). Glyoxal, glucose, glyoxylic acid, pyruvic acid and sodium cyanoborohydride ($NaCNBH_3$) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Dehydrated skim milk was purchased from Difco Lab. (Detroit, MI). A PD-10 column, cyanogenbromide (CNBr)-activated Sepharose 4B, Sephacryl S-200, and Sephadex G-15 were purchased from Pharmacia Biotech AB (Uppsala, Sweden). Alkaline phosphatase (AP)-conjugated sheep anti-rabbit immunoglobulin G (IgG) antibody was purchased from Boehringer Mannheim (Mannheim, Germany). Super Block Blocking Buffer in phosphate-buffered saline (PBS) and alkaline phosphatase substrate kits were purchased from Pierce Chemical Co. (Rockford, IL). Microtitration plates (96-well; High binding E.I.A./R.I.A. plates) were purchased from Corning Costar (Cambridge, MA). Centriprep-10 and PM-10 ultrafiltration membranes were purchased from Amicon, Inc. (Beverly, MA), while a polyvinylidene fluoride (PVDF) membrane was purchased from Atto Co. (Tokyo, Japan). All other chemicals were of the highest grade available from commercial sources.

Preparation of AGE Proteins, CML and CEL Protein

AGE-BSA and AGE-RSA were prepared as described previously (14). Briefly, each protein was incubated under sterile conditions with 0.1 M D-glyceraldehyde, glycolaldehyde, methylglyoxal or glyoxal and 5 mM DTPA in 0.2 M phosphate buffer (pH 7.4) at 37°C for 7 d and, then, low molecular weight reactants and aldehydes were removed using a PD-10 column and dialysis against PBS (pH 7.4). CML-BSA was prepared as described elsewhere (15). Briefly, 50 mg/ml protein was incubated at 37°C for 24 hr with 45 mM glyoxylic acid and 150 mM $NaCNBH_3$ in 2 ml of 0.2 M phosphate buffer (pH 7.4), followed by PD-10 column chromatography and dialysis against PBS. Carboxyethyllysine (CEL)-BSA was prepared as described elsewhere (16). Briefly, 50 mg/ml BSA was incubated at 37°C for 24 hr with 45 mM pyruvic acid and 150 mM $NaCNBH_3$ in 2 ml of 0.2 M phosphate buffer (pH 7.4), followed by PD-10 column chromatography and dialysis against PBS. Protein concentrations were determined with Dc protein assay reagent (Bio-Rad Laboratories, Richmond, CA) using BSA as a standard.

Preparation of Polyclonal Anti-AGE Antibodies

4 mg of various AGE-RSAs (incubated with D-glyceraldehyde, glycolaldehyde, methylgly-



Scheme 1. Possible routes of AGE formation. Advanced glycation end-products (AGE) arise from the decomposition of Amadori products (AGE-1), the glycolysis intermediate product glyceraldehyde (AGE-2), the Schiff base fragmentation product glycolaldehyde (AGE-3), the glyceraldehyde and the Amadori product fragmentation product methylglyoxal (AGE-4), and the autoxidation of glucose to glyoxal (AGE-5). CEL, N-(carboxyethyl)lysine; CML, N-(carboxymethyl)lysine.

oxal or glyoxal) were emulsified in 50% Freund's complete adjuvant and injected intradermally into rabbits. This procedure was repeated at weekly intervals for 6 weeks. After a 2-week pause, the rabbits were given a booster injection of 4 mg of each antigen. Animals were bled on the tenth day after this injection and serum was obtained for purification.

Purification of Non-CML AGE Antibodies from Polyclonal AGE Antibodies by Affinity Chromatography

Antibodies specific for non-CML AGE were isolated from the antiserum by affinity chromatography. Four kinds of AGE-BSA or CML-BSA (100 mg of each protein) were coupled to 20 ml of CNBr-activated Sepharose 4B according to the manufacture's instructions. 20 ml of rabbit serum was applied to a column (1.5 × 5.5 cm) of Sepharose 4B coupled with AGE-BSAs (incubated with D-glyceraldehyde, glycolaldehyde, methylglyoxal or glyoxal). After extensive washing with PBS, the adsorbed fractions were eluted with 20 mM sodium phosphate buffer containing 3 M potassium thiocyanate (pH 7.4). The AGE antibody fractions, were pooled, concentrated using Centriprep-10 (Amicon, Inc.), and passed through a PD-10 column (Pharmacia Biotech AB) equilibrated with PBS. The AGE antibodies thus obtained were then loaded onto a CML-BSA-Sepharose 4B column (1.5 × 5.5 cm), which was washed with 30 ml of PBS to obtain the unadsorbed fraction (non-CML AGE antibodies). The adsorbed fraction (CML antibody) was then eluted with 20 ml of 20 mM sodium phosphate buffer containing 3 M potassium thiocyanate (pH 7.4). Fractions (1.0 ml) were monitored for absorbance at 280 nm. The unadsorbed fractions were pooled, concentrated with Centriprep-10, and passed through a PD-10 column equilibrated with PBS for use in our study.

Enzyme-Linked Immunosorbent Assay (ELISA)

Measurement of AGE was performed with a competitive ELISA, as described previously (14). Briefly, test samples (50 μl) were added to each well as a competitor for 50 μl of non-CML AGE antibodies (1:250 - 1:1000), followed by incubation for 2 hr at room temperature with gentle shaking on a horizontal rotary shaker. Results were expressed as: B/B_0, calculated as (experimental OD − background OD)/(total OD − background OD).

The immunoreactivity of each fraction was read from the calibration curve (four kinds of AGE-BSAs) and was expressed as AGE units (U) per ml, with one unit corresponding to the amount of antibody reactive material found in AGE-BSA at a protein concentration of 1 μg/ml.

Size Distribution of Non-CML AGE in Serum from Diabetic Patients on Hemodialysis

50 ml of serum from 10 type 2 diabetic patients with end-stage renal disease on hemodialysis was subjected to ultrafiltration (PM-10 membrane, cut-off molecular weight (MW) 10 kDa, Amicon, Inc.) to separate the high molecular weight (HMW > 10 kDa) fraction from the low molecular weight (LMW < 10 kDa) fraction. The HMW fraction was applied to a Sephacryl S-200 column (1.5 × 110 cm), which was equilibrated with PBS (pH 7.4) and eluted with the same buffer (fraction size: 1.5 ml, flow rate: 10 ml/hr) in a cold room. The molecular weight markers used were aldolase (MW 160,000), BSA (MW 67,000), chymotrypsinogen A (MW 25,000), and vitamin B12 (MW 1,355). The LMW fraction was pooled, concentrated by lyophilization and dissolved in a small volume of distilled water. The precipitate was removed by centrifugation at 10,000 rpm for 10 min and the supernatant was applied to a Sephadex G-15 column (1.5 × 110 cm), which was equilibrated with 50 mM ammonium acetate buffer (pH 7.4) and eluted with the same buffer (fraction size: 1.5 ml, flow rate: 8 ml/hr) in a cold room. The molecular weight markers used were cytochrome c (MW 12,500), vitamin B_{12} (MW 1,355), and cytidine (MW 243). Each fraction was monitored for absorbence at 280 nm and the AGE activity of each fraction was measured by both AGE-ELISA and characteristic AGE-specific fluorescence (excitation maxima (Ex) = 360 nm/emission maxima (Em) = 440 nm). The immunoreactivity of each fraction was read from the calibration curve (four kinds of AGE-BSAs) and was expressed as AGE units (U) per ml, as described above.

Immunoblot Analysis

AGE-proteins and human serum protein samples were electrophoresed on a 7.5% SDS-gel

or 5-20% gradient SDS-gel. The proteins were transferred electrophoretically to PVDF membrane (Atto Co., Tokyo) for 45 min at 2.5 mA per cm^2. The membrane was blocked with 4% skim milk (Difco, MI) in PBS for 1 hr at room temperature, reacted for 2 hr with the immunoaffinity-purified antibodies (1:250-1:1000 diluted in 4% skim milk in PBS), washed three times for 5 min each with PBS-Tween 20 buffer, incubated for 1 hr in 4% skim milk in PBS with 1:2000 diluted anti-rabbit IgG coupled to alkaline phosphatase, washed five times for 5 min each with PBS-Tween 20, and finally, incubated with 5-bromo-4-chloro-3-indoyl phosphate-nitroblue tetrazolium.

Results

Characterization of the Immunogen

AGE-RSA during incubation for 7 d with glyceraldehyde, glycolaldehyde, methylglyoxal or glyoxal was monitored by AGE-specific fluorescence. Fluorescence was only associated with the AGE-RSA complex and was not seen in the control RSA after 7 d of incubation without these compounds. The excitation and emission maxima of the four types of AGE were 360–380 and 430–450 nm, respectively, in close agreement with glucose-modified AGE-RSA. The peak fluorescence intensity was similar to that of five kinds of AGE-proteins (Fig. 1A). SDS-PAGE of RSA incubated with these compounds is shown in Fig. 1B. No change was observed after incubation of RSA for 7 d without these compounds. AGE-RSA incubated with 0.5 M glucose for 8 weeks migrated much more slowly, yielding a broad band larger than 68 kD and suggesting that covalently linked adducts of RSA had been formed nonenzymatically. Very extensive crosslinking of RSA was seen after incubation with short chain sugars, glyceraldehyde and glycolaldehyde. In the case of dicarbonyl compounds (methylglyoxal and glyoxal), there was much less aggregate formation.

Separation of Non-CML AGE Antibodies from Polyclonal AGE Antibodies

The polyclonal AGE antisera obtained after incubation of AGE-RSA (with glyceraldehyde, glycolaldehyde, methylglyoxal, or glyoxal) were purified by four kinds of AGE-BSA affinity chromatography and then were separated by CML-BSA affinity chromatography (Fig. 2).

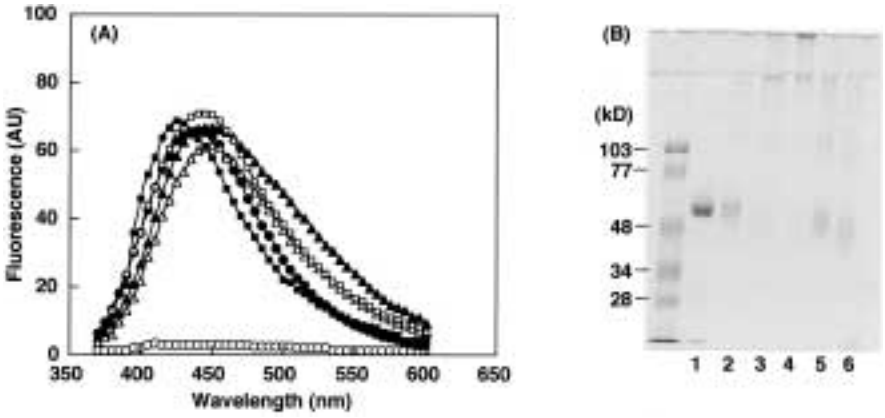


Fig. 1. Characteristics of rabbit serum albumin incubated with glyceraldehyde, glycolaldehyde, methylglyoxal and glyoxal (AGE-RSAs). Rabbit serum albumin (20 mg/ml) was incubated under sterile conditions with 0.1 M D-glyceraldehyde, glycolaldehyde, methylglyoxal or glyoxal and 5 mM DTPA in 0.2 M phosphate buffer (pH 7.4) at 37°C for 7 d and then low molecular weight reactants and aldehydes were removed using a PD-10 column and dialysis against phosphate-buffered saline (PBS, pH 7.4). (A) Fluorescence emission spectra of AGE-RSAs (1.0 mg/ml) at 360 nm after incubation with glyceraldehyde (Δ), glycolaldehyde (▲), methylglyoxal (□), glyoxal (■) or glucose (●), and after incubation without these compounds (○). The incubation time was 7 d, except for glucose (8 weeks). (B) SDS-PAGE of AGE-RSAs. Two micrograms of nonglycated RSA (without compounds, lane 1) and AGE-RSAs (incubated with glucose (lane 2), glyceraldehyde (lane 3), glycolaldehyde (lane 4), methylglyoxal (lane 5) and glyoxal (lane 6) were loaded onto a 7.5% polyacrylamide gel. Staining of the gel was done with Coomassie Brilliant Blue. Size markers (kD) are shown on the left.

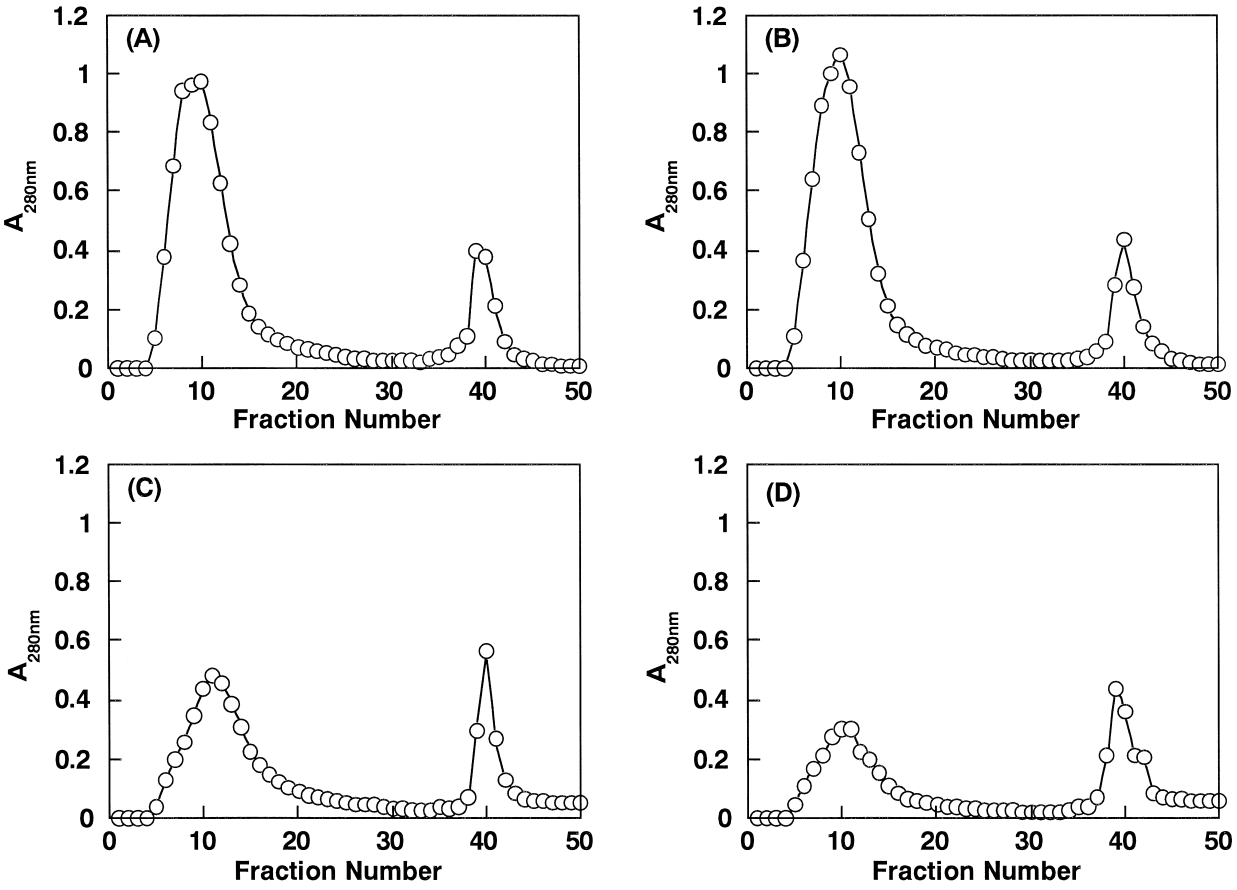


Fig. 2. Separation of non-CML AGE antibodies from polyclonal AGE antibodies by CML-BSA affinity chromatography. Affinity chromatography was performed with polyclonal anti-AGE-RSA incubation for 7 d with glyceraldehyde (A), glycolaldehyde (B), methylglyoxal (C) and glyoxal (D). Twenty milliliters of rabbit serum was applied to a column (1.5 × 5.5 cm) of Sepharose 4B coupled with AGE-BSAs (incubated with D-glyceraldehyde, glycolaldehyde, methylglyoxal or glyoxal). After extensive washing with PBS, the adsorbed fractions were eluted with 20 mM sodium phosphate buffer containing 3 M potassium thiocyanate (pH 7.4). The AGE antibody fractions were pooled, concentrated using Centriprep-10 (Amicon, Inc.) and passed through a PD-10 column (Pharmacia Biotech AB) equilibrated with PBS. The AGE antibodies obtained were then loaded onto a CML-BSA-Sepharose 4B column (1.5 × 5.5 cm), which was washed with 30 ml of PBS to obtain the unadsorbed fraction (non-CML AGE antibodies). The adsorbed fraction (CML antibody) was then eluted with 20 ml of 20 mM sodium phosphate buffer containing 3 M potassium thiocyanate (pH 7.4).

We calculated the proportion of antibodies bound to the CML affinity gel (eluted as the second peak in Fig. 2) to that of the antibodies unbound to CML affinity gel (eluted as at the first peak). Antibodies unbound to the CML affinity gel and eluted as the first peak (the non-CML AGE fraction) accounted for 83% of those obtained from AGE-RSA incubated with glyceraldehyde or glycolaldehyde (non-CML AGE-Ab-2 or -3, Figs. 2A and B), 71% of those obtained from AGE-RSA incubated with methylglyoxal (non-CML AGE-Ab-4, Fig. 2C), and 58% of those obtained from AGE-RSA incubated with glyoxal (non-CML AGE-Ab-5, Fig. 2D).

Characterization of Non-CML AGE Antibodies

Characterization of the non-CML AGE antibodies obtained by CML-BSA affinity chromatography was done using a competitive ELISA with several AGE-modified proteins (Fig. 3). Four types of non-CML AGE antibodies binding to each AGE-BSA were not altered by the addition of glycated-human serum albumin (HSA) (data not shown), indicating that early glycation products, such as Amadori products, were not the immunoreactive epitopes. We also investigated whether non-CML AGE antibodies purified by CML-BSA affinity chromatography could react with CML-BSA and CEL-BSA. CML-BSA and CEL-BSA did not inhibit all of

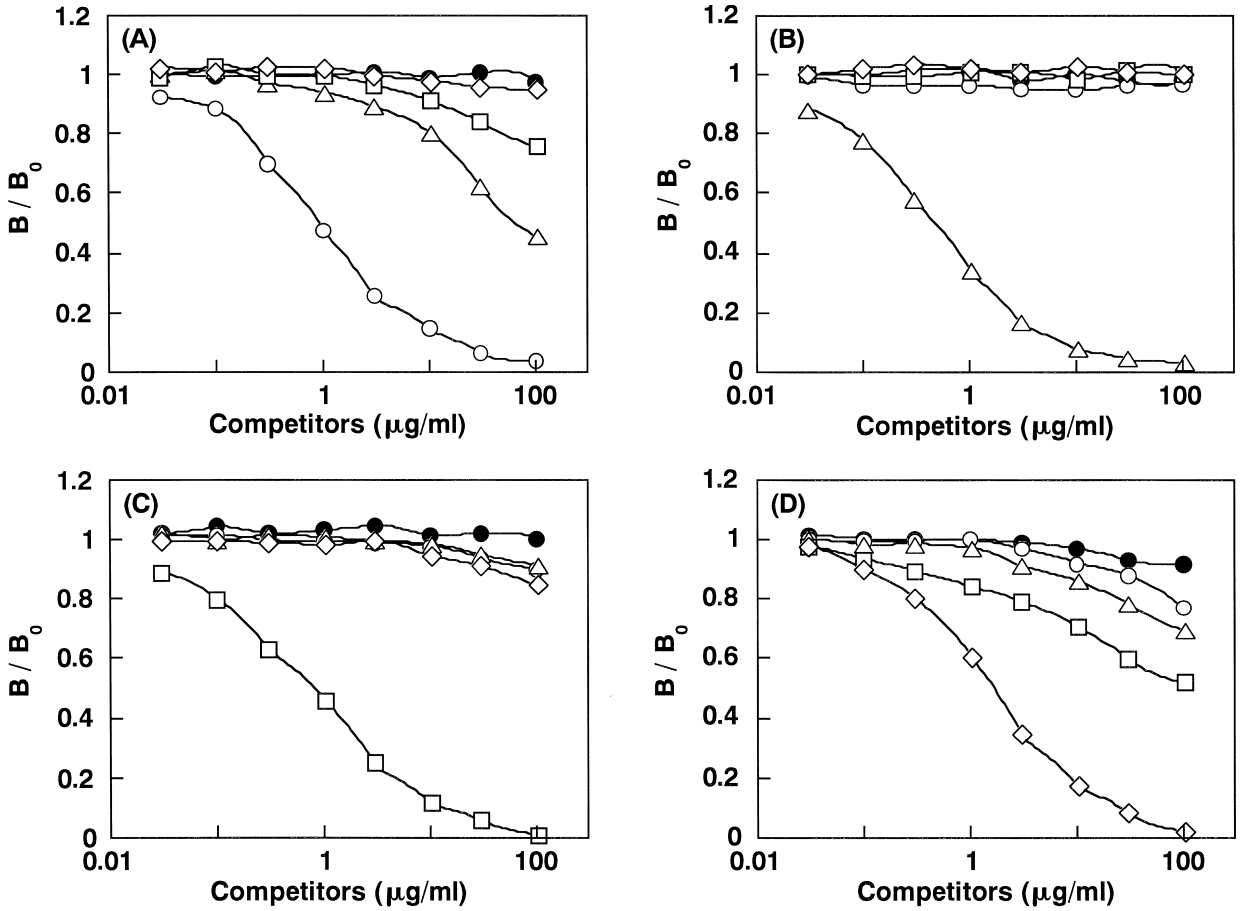


Fig. 3. Immunoreactivity of non-CML AGE antibodies with various AGE preparations. Non-CML AGE antibody was obtained by incubation of AGE-RSA with glyceraldehyde (A), glycolaldehyde (B), methylglyoxal (C) and glyoxal (D). BSA was modified by incubation with glucose (●), glyceraldehyde (○), glycolaldehyde (△), methylglyoxal (□), and glyoxal (◇).

the non-CML AGE antibodies from binding to each AGE-BSA (data not shown). Pentosidine-BSA and pyrraline-BSA also did not inhibit these non-CML AGE antibodies (data not shown). To determine which kinds of AGE were recognized by these non-CML AGE antibodies, BSA was incubated with glucose (AGE-1), glyceraldehyde (AGE-2), glycolaldehyde (AGE-3), methylglyoxal (AGE-4) and glyoxal (AGE-5). As shown in Fig. 3A, non-CML AGE-Ab-2 completely inhibited the binding of AGE-2 and also partially inhibited the binding of AGE-3. Non-CML AGE-Ab-3 and -4 specifically inhibited AGE-3 or AGE-4, respectively (Figs. 3B and C). Non-CML AGE-Ab-5 completely inhibited the binding of AGE-5 and partially inhibited the binding of AGEs-3 and -4 (Fig. 3D). However, AGE-1 did not inhibit all of the non-CML AGE antibodies from binding to AGE-2, -3, -4, or -5 (Figs. 3A–D). Immunoblot analysis with immunoaffinity-purified antibodies confirmed the results obtained by ELISA (Fig. 4). These findings indicated that AGE-BSA modified by short chain sugars and dicarbonyl compounds had the highest reactivity for non-CML AGE antibodies.

Size Distribution of AGE in Serum from Diabetic Patients on Hemodialysis

Using the affinity-purified non-CML AGE antibodies, we examined the size distribution of AGE in serum obtained from type 2 diabetic patients on hemodialysis. The size distribution of non-CML AGE was determined by Sephacryl S-200 column chromatography. Figure 5A shows the distribution of the high molecular weight fractions in serum from the diabetic patients monitored for absorbance at 280 nm and AGE-specific fluorescence. Figures 5B and C show the distribution detected by the four types of non-CML AGE antibodies ob-

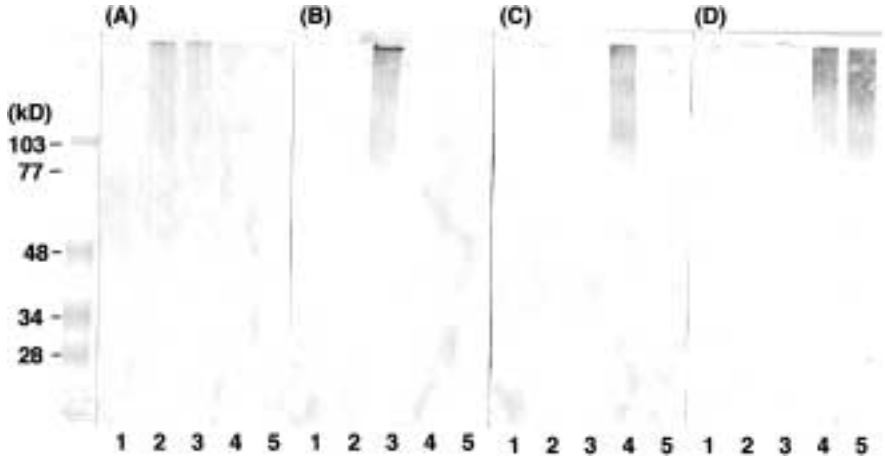


Fig. 4. Immunoblot analysis of various AGE-proteins with non-CML AGE antibodies. Aliquots (0.5 μg) of AGE-BSAs (incubated with glucose (lane 1), glyceraldehyde (lane 2), glycolaldehyde (lane 3), methylglyoxal (lane 4), and glyoxal (lane 5)) were loaded onto a 7.5% polyacrylamide gel. Formation of non-CML AGE in the protein was determined by immunoblot analysis using affinity-purified non-CML AGE antibodies. Non-CML AGE antibody was obtained by incubation of AGE-RSA with glyceraldehyde (A), glycolaldehyde (B), methylglyoxal (C) and glyoxal (D). Size markers (kD) are shown on the left.

tained after incubation of AGE-RSA with glyceraldehyde, glycolaldehyde, methylglyoxal and glyoxal, respectively. Non-CML AGE immunoreactive material was eluted as one peak with an apparent molecular weight of 200 kD. The levels of non-CML AGE-2 and -3 were much higher than those of non-CML AGE-4 and -5. Immunoblotting of serum protein samples with the above affinity-purified non-CML AGE antibodies showed major immunoreactivity with an apparent molecular weight of 200 kD. Minor immunoreactivity was observed with some high molecular weight proteins larger than albumin (Fig. 6). These findings supported ELISA results.

We also examined AGE in the low molecular weight fractions of serum from the diabetic patients using Sephadex G-15 column chromatography. Figure 7A shows the distribution of AGE in the low molecular weight fractions monitored for absorbance at 280 nm as well as for AGE-specific fluorescence. Figures 7B and C show the distribution detected with the affinity-purified non-CML AGE antibodies. Non-CML AGE immunoreactive material was eluted as two peaks by three types of non-CML AGE antibodies (obtained after incubation of AGE-RSA with glyceraldehyde, methylglyoxal and glyoxal) and these peaks contained species with an apparent molecular weight of 1.15 kD and 0.85 kD. On the other hand, non-CML AGE immunoreactive material was eluted as one peak by the non-CML AGE-Ab-3 obtained after incubation of AGE-RSA with glycolaldehyde and this peak contained AGE-peptide with an apparent molecular weight of 0.85 kD. The levels of non-CML AGE-2, -3, and -5 in the low molecular weight fraction were higher than that of non-CML AGE-4.

Discussion

The purpose of this study was to use specific antibodies to identify AGE-protein/-peptide modifications by short chain sugars (glyceraldehyde and glycolaldehyde) and dicarbonyl compounds (methylglyoxal and glyoxal) in diabetic patients. Methylglyoxal and glyoxal react with proteins by various pathways, leading to the formation of N-(carboxyalkyl) amino acids, imidazolones and imidazolium salts (6,13,16). Methylglyoxal is produced by non-enzymatic fragmentation of glyceraldehyde-3-phosphate (8) and glyoxal is formed by autoxidation of reducing sugars and polyunsaturated fatty acids (17,18). More recently, Odani et al. (19) reported that the methylglyoxal level was significantly higher in patients with uremia and diabetes, compared with age-matched healthy controls, while the glyoxal level in uremic plasma was significantly higher compared with that in diabetic and healthy controls. Recent in vitro studies showed that a significant

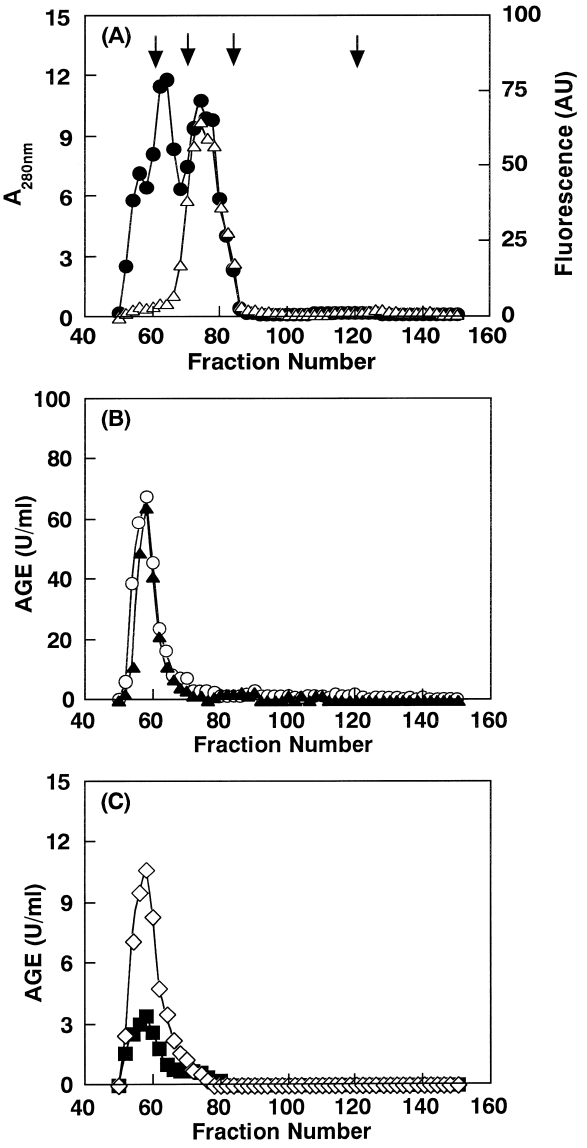


Fig. 5. Size distribution of high molecular weight AGE-proteins from diabetic patients on hemodialysis. High molecular weight protein fractions obtained from diabetic patients with end-stage renal disease on hemodialysis were subjected to gel filtration chromatography (Sephacryl S-200 column: 1.5 × 110 cm). The eluted fractions were monitored for non-CML AGE (glyceraldehyde, ○; glycolaldehyde, ▲; methylglyoxal, ■; glyoxal, ◇) by ELISA (data from triplicate determinations), AGE fluorescence (Ex = 360 nm / Em = 440 nm, △) and absorbance at 280 nm (●). Elution of aldolase (160,000 Da), BSA (67,000 Da) chymotrypsinogen A (25,000 Da) and vitamin B_{12} (1,355 Da) (molecular weight markers) is indicated by arrows. (A) Chromatography monitored by AGE fluorescence and absorbance at 280 nm. (B) Chromatography with affinity-purified non-CML AGE antibodies obtained by incubation of AGE-RSA with glyceraldehyde and glycolaldehyde. (C) Chromatography with affinity-purified non-CML AGE antibodies obtained by incubation of AGE-RSA with methylglyoxal and glyoxal.

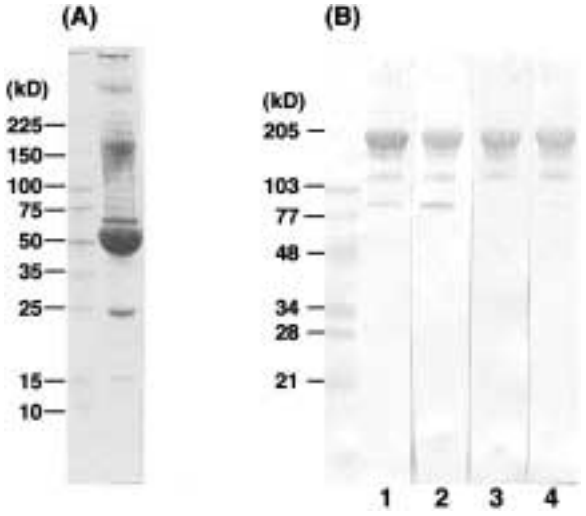


Fig. 6. Immunoblot analysis of serum from diabetic patients on hemodialysis with non-CML AGE antibodies. 20 μg of serum was treated with 1%-SDS in the absence of 2-mercaptoethanol and the sample was loaded onto 5–20% gradient polyacrylamide gel. (A) Detection of serum protein bands was performed with Coomassie Briliant Blue staining. (B) Formation of non-CML AGE was determined by immunoblot analysis using affinity-purified non-CML AGE antibodies. Non-CML AGE antibody was obtained by incubation of AGE-RSA with glyceraldehyde (lane 1), glycolaldehyde (lane 2), methylglyoxal (lane 3) and glyoxal (lane 4). Size markers (kD) are shown on the left.

level of glycolaldehyde was produced by cultured cells (20). Glycolaldehyde-modified proteins are known to undergo crosslinking in vitro (13). These short chain sugars and dicarbonyl compounds from AGE in vitro and have been postulated to be a major source of intracellular and plasma AGE. More recently, Shamsi et al. (21) reported that methylglyoxal-mediated protein modification occured during the Maillard reaction in vivo. Although the formation of AGEs by glyceraldehyde, glycolaldehyde and glyoxal is known to occur in vitro (6,13), there is still no evidence for such AGE found in vivo.

Our previous study showed that both CML and glucose-modified non-CML AGEs were present in serum. There is also evidence that non-CML AGEs, rather than CML AGEs, should be more closely investigated when studying the pathophysiology of AGE-related diseases (14). In the current study, RSA modified by incubation with short chain sugars (glyceraldehyde and glycolaldehyde) and dicarbonyl compounds (methylglyoxal and gly-

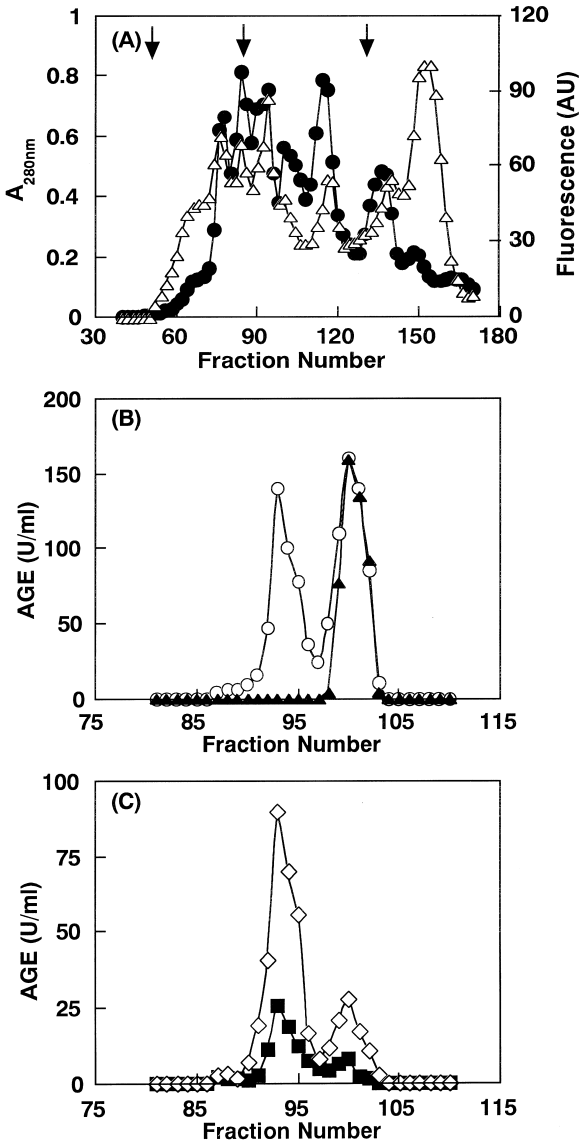


Fig. 7. Size distribution of low molecular weight AGE-peptides from diabetic patients on hemodialysis. Low molecular weight fractions obtained from diabetic patients with end-stage renal disease on hemodialysis were subjected to gel filtration chromatography (Sephadex G-15 column: 1.5 × 110 cm). The eluted fractions were monitored for non-CML AGE (glyceraldehyde, ○; glycolaldehyde, ▲; methylglyoxal, ■; glyoxal, ◇) by ELISA (data from triplicate determinations), AGE florescence (Ex = 360 nm/ Em = 440 nm, △) and absorbance at 280 nm (●). Elution of cytochrome c (V_0), vitamin B_{12} (1,355 Da) and cytidine (243 Da) (molecular weight markers) is indicated by arrows. (A) Chromatography monitored by AGE fluorescence and absorbance at 280 nm. (B) Chromatography with affinity-purified non-CML AGE antibodies obtained by incubation of AGE-RSA with glyceraldehyde and glycolaldehyde. (C) Chromatography with affinity-purified non-CML AGE antibodies obtained by incubation of AGE-RSA with methylglyoxal and glyoxal.

oxal) was used to immunize rabbits and a high titer of antiserum without any reactivity against the carrier proteins was obtained. We then produced four types of specific antibodies (non-CML AGE-Ab-2, -3, -4, and -5) that recognized non-CML AGE derived from short chain sugars and dicarbonyl compounds using immunoaffinity chromatography (Fig. 2). AGE-Ab-2 is the first reported AGE antibody that cross-reacts with proteins modified with glyceraldehyde, but not glycolaldehyde. AGE-Ab-3 is also the first reported AGE antibody that cross-reacts with protein treated with glycolaldehyde alone. AGE-Ab-4 cross-reacts with protein treated with methylglyoxal alone. Uchida et al. (22) and Oya et al. (23) recently reported that their antibodies directed against methylglyoxal-derived KLH cross-reacted with protein modified with methylglyoxal, as well as glyceraldehyde or glycolaldehyde. Shamsi et al. (21) reported that antibodies directed against methylglyoxal-derived–RNase cross-reacted with N-acetylarginine modified with methylglyoxal or sugars, such as glyceraldehyde, fructose and ribose. Thus, it seems likely that the epitope of non-CML AGE-Ab-4 is different from those of previously reported antibodies. AGE-Ab-5 is also the first reported anti-AGE antibody that cross-reacts with proteins modified with glyoxal, methylglyoxal or glycolaldehyde. The epitope of the non-CML AGE antibodies appears to differ from pentosidine, pyrraline, CML and CEL, because BSA preparations conjugated with these compounds are not recognized by these antibodies (data not shown).

We next examined whether four types of non-CML anti-AGE antibodies could detect non-CML AGEs in vivo. In the clinical measurement of AGE, the most convenient sample to test is blood, so we used an ELISA and immunoblotting to detect four kinds of non-CML AGE in serum. We examined the size distribution of immunoreactive AGEs in serum samples obtained from type 2 diabetic patients on hemodialysis. The estimated weight of high molecular weight AGE protein(s) detected by a previous AGE-ELISA system using a specific antibody (non-CML AGE-Ab-1) for glucose-derived non-CML AGE, was 200 kD and 65 kD (14). In the current study, four types of non-CML AGE immunoreactive materials eluted as one peak with an apparent molecular weight of 200 kD (Fig. 5). Immunoblotting of serum protein samples with these non-CML AGE anti-

bodies showed major immunoreactivity with an apparent molecular weight of 200 kD (Fig. 6). Shamsi et al. (21) reported that western blotting of diabetic serum proteins samples with their immunoaffinity-purified antibody to methylglyoxal-modified RNase showed major immunoreactivity with albumin; whereas, our non-CML AGE-Ab-4 showed major immunoreactivity with a higher molecule weight protein (MW = 200 kD) by ELISA and immunoblotting. It seems likely that the epitope of non-CML AGE-Ab-4 is different from those of previously reported antibodies. We also examined AGE in the low molecular weight fractions of serum from the diabetic patients and found that non-CML AGE-Ab-2, -4 and -5 immunoreactive material was eluted as two peaks with apparent molecular weights of 1.15 and 0.85 kD (Fig. 7). In our previous study (14), non-CML AGE-Ab-1 immunoreactive material was eluted as two peaks with the same molecular weights; whereas, non-CML AGE-Ab-3 immunoreactive material was eluted as one peak with an apparent molecular weight of 0.85 kD (Fig. 7). These four types of non-CML AGE antibodies enable us to identify compounds created by the Maillard reaction in vivo.

Today, there is general agreement that there are multiple sources and mechanisms for the formation of AGE in vivo, involving oxidative and nonoxidative chemistry of reducing sugars, Schiff bases, Amadori adducts and metabolic intermediates such as glyceraldehyde and methylglyoxal (24–26). The biological and physiological significance of glycolaldehyde and glyoxal are unclear, but glyoxal may be an important marker of diabetic complications (24,25). We now provide direct immunochemical evidence for the coexistence of five kinds of non-CML AGE structures within AGE-protein(s)/-peptide(s) in the blood. We propose that various types of non-CML AGE are formed by the Maillard reaction, sugar autoxidation and sugar metabolic pathways in vivo, as shown in Scheme 1.

In summary, we prepared specific antibodies recognizing four types of non-CML AGEs derived from short chain sugars (glyceraldehyde and glycolaldehyde) and dicarbonyl compounds (methylglyoxal and glyoxal). These antibodies revealed four classes of non-CML AGEs in circulating AGE-protein(s)/-peptide(s) from diabetics, showing that short chain sugar- and dicarbonyl compound–mediated protein modification occurs during the Maillard reaction in vivo. This is an important step toward elucidationing how such modifications may influence the complications of diabetes.

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