

# Autoantibody Against N<sup>ε</sup>-(Carboxymethyl)lysine

## An Advanced Glycation End Product of the Maillard Reaction

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**Prolonged incubation of proteins with reducing sugar produces advanced glycation end products (AGEs), which are implicated as factors for aging and diabetic complications. We previously demonstrated the presence of N<sup>ε</sup>-(carboxymethyl)lysine (CML), one of the main AGE structures, in human and animal tissues using a monoclonal anti-CML antibody (6D12). These findings suggest that CML structures present in vivo could serve as immunogens to generate autoantibodies. This suggestion was tested in the present study. First, plasma samples from diabetic rats reacted positively with AGE bovine serum albumin (BSA). These reactivities increased with the duration of diabetic states and were inhibited specifically by CML-BSA. Second, a fraction purified from plasma of diabetic patients, which bound to AGE-BSA, showed a positive reaction to CML-BSA and furthermore also to human lens proteins, which are known to undergo CML modification in vivo. Finally, patients with renal failure caused by diabetes or nondiabetic pathologies had a higher autoantibody activity against CML structure than that in normal subjects or diabetic patients without renal failure. These results indicate that CML accumulated in vivo serves as an immunological epitope to generate an autoantibody specific for CML that might be used as a potential marker for diabetic nephropathy or chronic renal failure. *Diabetes* 48:1842–1849, 1999**

**L**ong-term incubation of proteins with glucose leads, through the formation of early products such as Schiff base adducts and Amadori rearrangement products, to the formation of advanced glycation end products (AGEs), which are characterized by fluorescence, brown color, and inter- and intramolecular cross-linking (1,2). AGE structures reported so far include fluorescent and cross-linked structures such as

pentosidine (3), crosslines (4), and imidazolones (5,6), and nonfluorescent and non-cross-linked structures such as N<sup>ε</sup>-(carboxymethyl)lysine (CML) (7) and pyrroline (8). Immunological studies using anti-AGE antibodies have confirmed the presence of AGEs in animal and human tissues (9–25). In 1991, we prepared a monoclonal anti-AGE antibody (6D12) that reacted with AGE products such as those derived from bovine serum albumin (BSA), human serum albumin, and hemoglobin, but not with unmodified proteins, suggesting that a common structure(s) could serve as one of the major epitopes expressed in AGE preparations (26). Subsequent studies from our laboratory showed that 6D12 recognizes CML protein adduct as an epitope (27). Furthermore, immunological studies using 6D12 as well as other anti-AGE antibodies with unknown epitopes have identified the accumulation of AGE proteins in several human and animal tissues in association with aging (9,10) and age-related disease states such as diabetic nephropathy (11–13), diabetic retinopathy (14,15), atherosclerosis (16–18), hemodialysis-related amyloidosis (19,20), Alzheimer's disease (21–23), actinic elastosis in photo-enhanced skins (24), and peritoneum of continuous ambulatory peritoneal dialysis (25). These findings allowed us to postulate that AGE structures present in vivo could serve as an immunological epitope to raise autoantibodies against AGE structures, particularly CML.

This possibility was tested in the present study in diabetic rats and patients with several diseases. Experiments with streptozotocin (STZ)-induced rats showed the presence of autoantibodies against AGE structures, particularly those against CML; the activity of the autoantibody increased with the duration of diabetic states. Experiments in diabetic patients also indicated the presence of the autoantibodies in the plasma against CML structure. Furthermore, the autoantibody against CML structure was higher in patients with renal failure than in normal subjects or diabetic patients without renal failure. These results suggest that accumulation of CML in vivo serves as an immunological epitope to raise an autoantibody against CML, suggesting a possible role in the development of diabetic nephropathy or chronic renal failure.

### RESEARCH DESIGN AND METHODS

**Materials.** BSA (fraction V) and STZ were purchased from Sigma (St. Louis, MO). D-Glucose, glyoxylic acid, and NaCNBH<sub>3</sub> were purchased from Wako (Osaka, Japan). Phosphate-buffered saline (PBS) (–) was purchased from Nissui Pharmaceutical (Tokyo). Biotinylated preparations of goat anti-human IgG antibody were purchased from Vector Laboratories (Burlingame, CA). Peroxidase-linked sheep anti-rat IgG antibody and anti-mouse IgG antibody were purchased from Amersham (Buckinghamshire, U.K.). Peroxidase-linked rabbit anti-human IgG, IgM, and IgA immunoglobulins were purchased from MBL (Nagoya, Japan). The 96-well microtitration plates (Nunc Immunoplate II) were purchased from Nalge Nunc International (Roskilde, Denmark). Formylcellulofine gels were purchased from Seikagaku Kogyo (Tokyo).

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AGE, advanced glycation end product; BSA, bovine serum albumin; CEL, N<sup>ε</sup>-(carboxyethyl)lysine; CML, N<sup>ε</sup>-(carboxymethyl)lysine; ELISA, enzyme-linked immunosorbent assay; FFI, 2-(2-furoyl)-4-(5)-(2-furanyl)-1H-imidazole; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; STZ, streptozotocin; WHHL, Watanabe heritable hypercholesterolemia.

**Animals.** Male Wistar rats (6-week-old) weighing 150 g were divided at random into control rats and diabetic rats. Diabetes was induced by an intravenous injection of 50 mg STZ/kg body wt in 0.01 mol/l citrate buffer (pH 4.3). Both groups of rats were provided with water and food ad libitum. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of our institutions. The animals were killed at 1, 3, 5, 14, or 29 weeks under ether anesthesia. Table 1 shows the characteristics of rats used in this study; diabetic rats at 1–29 weeks after STZ injection had a significantly lower body weight but a higher plasma glucose level than age-matched control rats.

**Preparation of human lens protein.** Water-soluble fractions were prepared from an intact lens of normal humans (one lens of a child <4 years of age and two lenses from 25- and 89-year-old individuals) as described previously (9). Protein concentrations were determined by the dye-staining method (Bio-Rad, Hercules, CA) or the bicinchoninic acid (BCA) method (Pierce, Rockford, IL). Studies involving human tissues were approved by the Ethics Review Committee for Human Experimentation of our institutions.

**Human plasma samples.** Human plasma samples were obtained from six groups (each group containing 10 individuals). The first group consisted of normal subjects aged  $72.8 \pm 2.9$  years (mean  $\pm$  SE). The second group consisted of diabetic patients with no significant renal failure (age  $63.2 \pm 3.8$  years,  $HbA_{1c}$   $8.4 \pm 0.3\%$ ). The third group consisted of diabetic patients with microalbuminuria (age  $68.5 \pm 4.6$  years,  $HbA_{1c}$   $8.8 \pm 0.3\%$ ). The fourth group consisted of diabetic patients with macroalbuminuria (age  $63.5 \pm 6.3$  years,  $HbA_{1c}$   $9.4 \pm 0.6\%$ ). The fifth group consisted of patients with diabetes with chronic renal failure who were under hemodialysis therapy. Patients in this group were  $67.7 \pm 6.6$  years old with a mean duration of hemodialysis of  $2.5 \pm 1.3$  years and mean  $HbA_{1c}$  of  $6.6 \pm 0.7\%$ . The sixth group consisted of nondiabetic patients who required hemodialysis treatment for chronic renal failure because of other nondiabetic causes. The mean age of this group was  $64.7 \pm 5.6$  years and the mean duration of hemodialysis was  $3.6 \pm 1.3$  years. Blood samples from hemodialysis patients were obtained immediately before hemodialysis therapy and stored at  $-80^\circ\text{C}$  until use in experiments.

**Purification of human autoantibody against AGE structure.** Formylcellulofine gels were coupled to BSA or AGE-BSA as described previously (28). All procedures for purification were performed at  $4^\circ\text{C}$ . Human plasma (20 ml) from eight diabetic patients was passed over a column ( $1.0 \times 10$  cm) of formylcellulofine-BSA gel in PBS. The non-adsorbed fraction was then passed over to another set of the same column to remove antibody populations reactive to BSA. The non-adsorbed fractions were then combined and applied to a column ( $1.5 \times 10$  cm) of formylcellulofine coupled with AGE-BSA. After extensive washing with PBS, the adsorbed fractions were eluted with 50 ml of 0.1 mol/l glycine-HCl buffer (pH 3.0). Eluted sample fractions were combined, neutralized, and applied to another set of a column of BSA-conjugated formylcellulofine gels to completely remove BSA-reactive antibodies. The non-adsorbed fractions from this column were pooled, concentrated, dialyzed against PBS, and used as the human autoantibody to AGE proteins. A portion of the final sample obtained from diabetic patients was subjected to an immunodiffusion quantitation kit using antibodies to human IgG, IgM, and IgA (Wako). The results showed that the contents of immunoglobulin were 69% IgG, 18% IgM, and 13% IgA.

**Mini-scale preparation of individual human autoantibody fractions against AGE structure.** Plasma (0.1 ml) from each individual of the six groups described above was mixed with 1.0 ml of BSA-conjugated formylcellulofine gels (50% suspension in PBS) in a 1.5-ml microcentrifuge tube. The mixture was shaken overnight at  $4^\circ\text{C}$  and centrifuged at 15,000 rpm for 30 s at  $4^\circ\text{C}$ . The supernatant (0.55 ml) was then mixed with 0.5 ml of AGE-BSA-conjugated formylcellulofine gels, followed by shaking for 6 h at  $4^\circ\text{C}$ . The gels were washed five times with 1 ml of PBS and suspended in 0.5 ml of 0.1 mol/l glycine-HCl buffer (pH 2.3) for 30 min at  $4^\circ\text{C}$ . The mixture was then centrifuged, and the supernatant was collected and neutralized with 1 mol/l Tris buffer (pH 9.5). To further remove antibodies reactive to BSA, the sample was then added to 0.5 ml BSA-conjugated formylcellulofine gels. After shaking overnight at  $4^\circ\text{C}$  followed by a brief cen-

trifugation, the supernatant was collected and used for the noncompetitive enzyme-linked immunosorbent assay (ELISA) as human autoantibodies against AGE structure.

**Preparations of monoclonal anti-AGE antibody.** The monoclonal anti-AGE antibody was prepared and characterized as reported previously (26). Briefly, splenic lymphocytes from Balb/c mice immunized with AGE-BSA were fused to myeloma P3U1 cells. The hybrid cells were screened, and two cell lines that had a positive reaction to AGE-human plasma albumin but a negative reaction to BSA were selected through successive subcloning. Each antibody was produced in ascites fluid of Balb/c mice and further purified by protein A sepharose column chromatography. One of these antibodies, designated as 6D12, was used in the present study.

**Preparation of AGE-BSA and CML-BSA.** AGE-BSA was prepared as described previously (28). Briefly, BSA (1.6 g) was dissolved with 3.0 g D-glucose in 10 ml of 0.5 mol/l sodium phosphate buffer (pH 7.4). The solution was sterilized by ultrafiltration (0.45  $\mu\text{m}$ /filter), incubated at  $37^\circ\text{C}$  for 9 months, and dialyzed against 20 mmol/l sodium phosphate buffer (pH 7.4) containing 0.15 mol/l NaCl. As a control for AGE-BSA, BSA was incubated in parallel without glucose. CML-BSA was prepared as described previously (27,30). Briefly, 2 mg/ml of BSA was incubated at  $37^\circ\text{C}$  for 24 h with 0.75 mol/l glyoxylic acid and 0.3 mol/l NaCNBH<sub>3</sub> in 0.5 mol/l sodium phosphate buffer (pH 7.4), followed by dialysis against PBS. As a control for CML-BSA, BSA was incubated in parallel without glyoxylic acid and NaCNBH<sub>3</sub>. Amino acid analyses (27) revealed that CML contents of AGE-BSA and CML-BSA thus prepared were 7.69 and 34.47 mol of CML per mole of BSA, respectively.

**AGE determination in human lens proteins by ELISA.** Noncompetitive ELISA was performed as described previously (26). Briefly, each well of 96-well immunoplate was coated with 0.1 ml of the sample (from 10 ng to 10  $\mu\text{g}/\text{ml}$ ) to be tested in 50 mmol/l carbonate buffer (pH 9.7) and maintained overnight at  $4^\circ\text{C}$ , followed by washing three times with PBS containing 0.05% Tween 20 (buffer A). Each well was then blocked with 0.2 ml of 0.5% gelatin in 50 mmol/l carbonate buffer (pH 9.7), washed with buffer A, and incubated with 0.1 ml of 6D12 (1  $\mu\text{g}/\text{ml}$ ) in buffer A containing 0.1% BSA for 2 h at room temperature. Wells were then washed and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody for 30 min, followed by reaction with 1,2-phenylenediamine dihydrochloride. The reaction was terminated by adding 1 mol/l sulfuric acid, followed by reading the absorbance at 492 nm on a micro-ELISA plate reader (Titertek Multiskan PLUS MKII). All ELISAs were performed for control in the absence of a primary antibody rather in the presence of a secondary antibody alone.

**Characterization of human and rat autoantibody.** Noncompetitive ELISA was performed using rat plasma and the purified human autoantibody fraction to detect immunoreactivities to AGE-BSA and CML-BSA. Each well was coated with 0.1 ml of 10  $\mu\text{g}/\text{ml}$  AGE-BSA or CML-BSA, blocked with gelatin and washed with buffer A. We added 0.1 ml of rat plasma (from 2  $\mu\text{g}$  to 5 mg/ml) or the purified human autoantibody fraction (from 80 ng to 20  $\mu\text{g}/\text{ml}$ ) to each well. After standing for 2 h at room temperature, the wells were washed, and the amount of the rat autoantibody bound to wells was determined by HRP-conjugated anti-rat IgG antibody. The amount of human autoantibody was determined by biotinylated anti-human IgG antibody, followed by reaction of avidin-biotin HRP complexes. HRP-conjugated anti-human IgG, IgM, and IgA antibodies were used to evaluate the contribution of human IgG, IgM, and IgA to autoantibody activity. The specificity of rat and human autoantibodies to AGE and CML structures was analyzed by competitive ELISA. Competitive ELISA was performed as described previously (26). Briefly, after coating with AGE-BSA or CML-BSA and blocking, each well was incubated for 2 h with 0.1 ml of rat plasma (1 mg/ml) or the purified human autoantibody fraction (1  $\mu\text{g}/\text{ml}$ ) in the presence of various concentrations of AGE-BSA or CML-BSA (0–1 mg/ml). Each well was washed and reacted with HRP-conjugated anti-rat IgG or anti-human IgG antibody, followed by determination of autoantibodies in the same way as described above. The results were expressed as  $B/B_0$ , where B represented the amount of anti-rat IgG or anti-human IgG antibody bound to the well in the presence of competitors while  $B_0$  was that bound to the well in the absence of competitors.

TABLE 1  
Characteristics of STZ-induced diabetic and age-matched control rats

Weeks	Body weight (g)		Plasma glucose (mg/dl)	
	Control	Diabetic	Control	Diabetic
1	180.0 $\pm$ 2.9 (7)	158.5 $\pm$ 4.8 (7)*	133.4 $\pm$ 10.1 (7)	401.5 $\pm$ 10.3 (7)†
5	259.7 $\pm$ 7.1 (6)	157.2 $\pm$ 3.9 (24)†	157.0 $\pm$ 11.5 (6)	556.0 $\pm$ 8.7 (24)†
14	324.3 $\pm$ 21.3 (7)	131.2 $\pm$ 5.9 (7)*	159.4 $\pm$ 18.6 (7)	574.4 $\pm$ 21.3 (7)†
29	378.0 $\pm$ 7.2 (7)	139.5 $\pm$ 13.4 (7)†	139.4 $\pm$ 5.7 (7)	514.7 $\pm$ 24.5 (7)†

Data are means  $\pm$  SE (*n*). \**P* < 0.01, †*P* < 0.001 vs. age-matched control.

**Specific reactivity of plasma of diabetic rats to AGE-BSA and CML-BSA.** The reactivity of each of 24 plasma samples from diabetic rats after 5 weeks of STZ injection and those of 6 age-matched control rats to AGE-BSA and CML-BSA was determined by noncompetitive ELISA. Briefly, each well was coated with 0.1 ml of AGE-BSA, CML-BSA, or BSA (10 µg/ml) and reacted with 0.1 ml of diluted plasma samples. The amount of rat autoantibody bound to each well was determined by HRP-conjugated anti-rat IgG antibody as described above. The linear range of the plasma protein concentrations for the assay was 0.5–5 mg/ml, and a representative plasma concentration of 1 mg/ml was used for the repeated analysis. Our preliminary experiments indicated that BSA gave a minimal background level when compared with other proteins such as rat serum albumin, human hemoglobin, human serum albumin, Rnase, and bovine collagen. However, because some plasma samples gave nonspecific background binding to the BSA-coated solid phase, the specific reactivity of each plasma to AGE-BSA was calculated by subtracting its reactivity to BSA from that to AGE-BSA. Similarly, the specific reactivity of each plasma to CML-BSA was calculated by subtracting its reactivity to BSA from that to CML-BSA and expressed as a difference in absorbance at 492 nm ( $\Delta 492$  nm).

**Immunoreaction of the purified human autoantibody fraction to human lens proteins.** This immunoreaction was performed by noncompetitive ELISA. Briefly, each well was coated with 0.1 ml of each water-soluble lens sample (10 µg/ml) and reacted with the purified human autoantibody fraction (0.3–3 µg/ml) or 6D12 (0.03–1 µg/ml). The amount of human autoantibody bound to lens proteins was determined by biotinylated anti-human IgG antibody, whereas that of 6D12 bound to lens proteins was determined by HRP-conjugated anti-mouse IgG antibody in a manner similar to that described above. The specificity of the purified human autoantibody fraction to lens sample was analyzed by competitive ELISA. Briefly, after coating with lens sample, each well was reacted with the purified human autoantibody fraction (3 µg/ml) or 6D12 (1 µg/ml) in the presence of CML-BSA (1 mg/ml). The amount of human autoantibody bound to lens proteins was determined as described above.

**Immunoreaction of purified human autoantibody fractions to AGE structure.** Human autoantibody fractions from samples of each individual of the six groups described above were purified as described earlier. Immunoreaction was performed by noncompetitive ELISA. Briefly, each well was coated with 0.1 ml of AGE-BSA or BSA (10 µg/ml) and reacted with 0.1 ml of each human autoantibody fraction (original, twofold, and fourfold diluted samples), followed by detection with biotinylated anti-human IgG antibody as described above. Because some human autoantibody fractions showed nonspecific binding to BSA-coated solid phase, which could not be inhibited completely with pretreatment of the excess amount of BSA, the specific reactivity of each human autoantibody fraction to AGE-BSA was calculated by subtracting its reactivity to BSA from that to AGE-BSA and expressed as a difference in absorbance at 492 nm in the same way as described for that autoantibody ( $\Delta 492$  nm).

**Amino acid analysis.** CML contents of modified proteins were quantitated by amino acid analysis as described (27,29). After acid hydrolysis with 6 N HCl for 24 h at 110°C, samples were subjected to an amino acid analyzer (Model 835; Hitachi, Tokyo), using an ion exchange high-performance liquid chromatography (HPLC) column (#2622 SC, 4.6 × 60 mm; Hitachi) and a ninhydrine post-column detecting system. Hippuryl-CML was prepared by incubation hippuryllysine with glyoxylic acid and NaCNBH<sub>3</sub> as described (27) and used as a standard. The identity of CML detected by HPLC was confirmed by fast atom bombardment (FAB) spectroscopy.

**Statistical analysis.** Data were expressed as means ± SE. Differences between groups were analyzed with unpaired Student's *t* test. Statistical significance was indicated by *P* < 0.05.

**RESULTS**

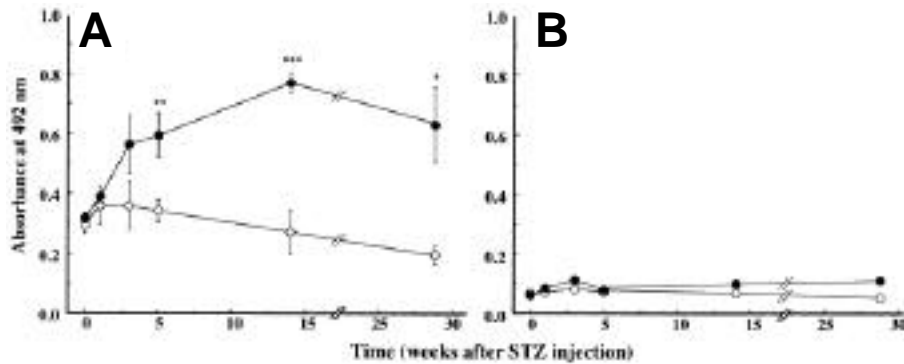
**Autoantibody against AGE-BSA in diabetic rat plasma.**

To examine whether the autoantibody to AGE structure was

produced in diabetic rats, plasma samples were prepared both from STZ-induced diabetic and age-matched control rats, followed by determination of their reactivity to AGE-BSA and BSA. The reactivity of plasma IgG to AGE-BSA increased significantly at 5 weeks after STZ injection and increased steadily up to 14 weeks after the injection, followed by a slight decrease at 29 weeks after injection, whereas the reactivity of plasma IgG of age-matched control to AGE-BSA remained at a basal level throughout the experiment (Fig. 1A). On the other hand, the reactivities of diabetic and control plasma IgG to native BSA remained at basal level and did not increase at all during the length of experiments (Fig. 1B). Similar results were obtained when AGE-BSA and BSA were replaced by AGE-rat serum albumin and rat serum albumin, respectively (data not shown), suggesting that the autoantibody against an AGE structure(s) could be generated under the diabetic conditions.

**Immunoreactivity of plasma of diabetic rats to CML-BSA.** CML structure is one of the main AGE structures present in vivo (30). To examine the possible generation of an autoantibody against CML structure in diabetic rats, immunoreactivity to CML-BSA was determined in the plasma. The plasma IgG of a 5-week diabetic rat showed a positive reaction towards AGE-BSA in a dose-dependent manner (Fig. 2A). And this reactivity to AGE-BSA was completely inhibited upon preincubation with an excess amount of AGE-BSA and CML-BSA, but not by BSA (Fig. 2B). Under these conditions, the same plasma IgG of diabetic rat also reacted with CML-BSA in a dose-dependent manner, but not with BSA, while no significant reactivity was noted in a plasma sample from an age-matched control (Fig. 2C). The reactivity to CML-BSA was inhibited by pretreatment with an excess amount of either AGE-BSA (>80%) or CML-BSA (>90%), but not by BSA (Fig. 2D). These results suggest the presence of an autoantibody against CML structure in the diabetic plasma. This notion was further examined by determining the autoantibody activity in plasma samples from 24 diabetic rats after 5 weeks of STZ injection. The autoantibody activities in these plasma samples as well as those from control rats are expressed as specific reactivity in Fig. 3. The mean specific reactivity to AGE-BSA in diabetic samples was 2.2-fold higher than that of control rat plasma samples (Fig. 3A). Similarly, the specific reactivities in these samples of diabetic rats to CML-BSA were 2.7-fold higher than the control levels (Fig. 3B). These results suggest that one main possible epitope of the autoantibody against AGE structure detected in plasma of diabetic rats is CML or its related structure.

**Characterization of human autoantibody against AGE structure.** We next examined whether a similar autoantibody



**FIG. 1.** Immunoreactivity of plasma of diabetic rats to AGE-BSA and BSA. Plasma samples from each diabetic rat (●) and age-matched control rats (○) were collected at 0, 1, 3, 5, 14, or 29 weeks after STZ injection, and their immunoreactivities to AGE-BSA (A) and BSA (B) were analyzed by noncompetitive ELISA. Data are means ± SE. Sample numbers (*n*) in each group corresponded to those described in Table 1, except that the sample number of 0- and 3-week diabetic rats was 7. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001 vs. age-matched control.

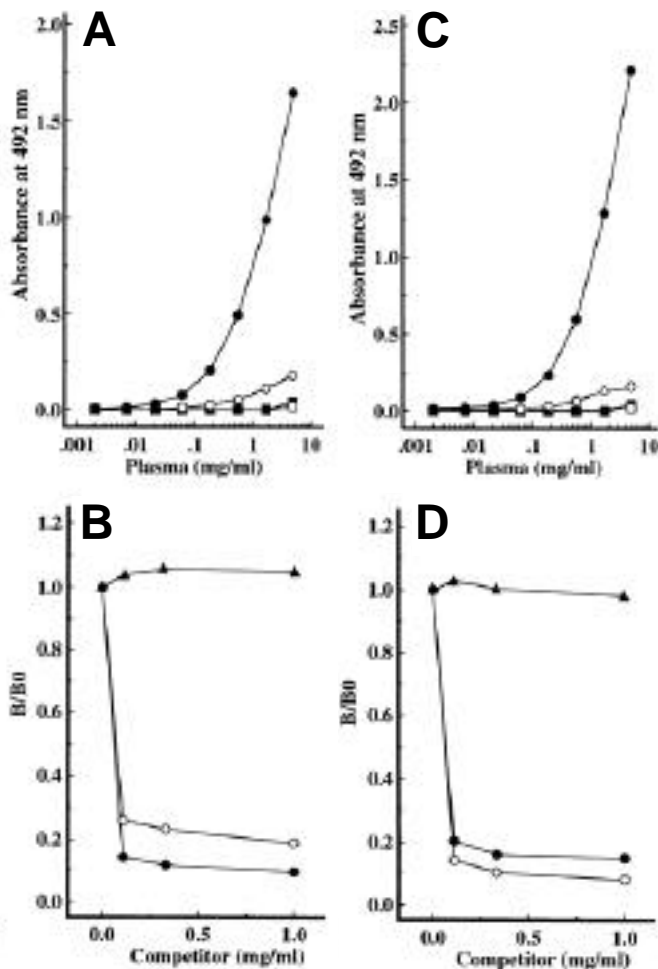


FIG. 2. Immunoreactivity of plasma of diabetic rats to CML-BSA and AGE-BSA. **A:** Reaction of a plasma sample from a 5-week diabetic rat to AGE-BSA (●) and BSA (■) was determined by a noncompetitive ELISA system. As a control, plasma from an age-matched control rat was examined for its reactivity to AGE-BSA (○) and BSA (□). **B:** Effects of AGE-BSA (●), CML-BSA (○), and BSA (▲) on immunoreaction of the same plasma sample used in **A** with AGE-BSA examined by a competitive ELISA. The results are expressed as ratio  $B/B_0$ , where  $B$  is the binding of anti-rat IgG antibody in the presence of competitors and  $B_0$  is that bound in the absence of a competitor. **C:** Under the noncompetitive ELISA system identical to that in **A**, we determined the reaction of the same plasma sample used in **A** to CML-BSA (●) and BSA (■). Control plasma was also examined for its reactivity to CML-BSA (○) and BSA (□). **D:** Under the competitive ELISA system identical to that in **B**, we determined AGE-BSA (●), CML-BSA (○), and BSA (▲) for their effects on the reaction of the same plasma of diabetic rats to CML-BSA. Results are expressed in a manner similar to that in **B**. Each data point was the mean of triplicate analyses.

could be detected in the human plasma. For this purpose, we initially screened 24 plasma samples from diabetic patients by an assay identical to Fig. 3 and found that several samples exhibited a significant reactivity to AGE-BSA. Using pooled plasma samples of eight of these diabetic patients, we affinity-purified the fraction that bound to a AGE-BSA column but not a BSA column and used it as the purified autoantibody fraction. IgG of this purified fraction reacted positively not only with AGE-BSA but also with CML-BSA in a dose-dependent manner, whereas the reaction to BSA was weak (Fig. 4A). The specific reactivity of IgM to AGE-BSA in this fraction was only 30% of the total activity, whereas that of IgA was negli-

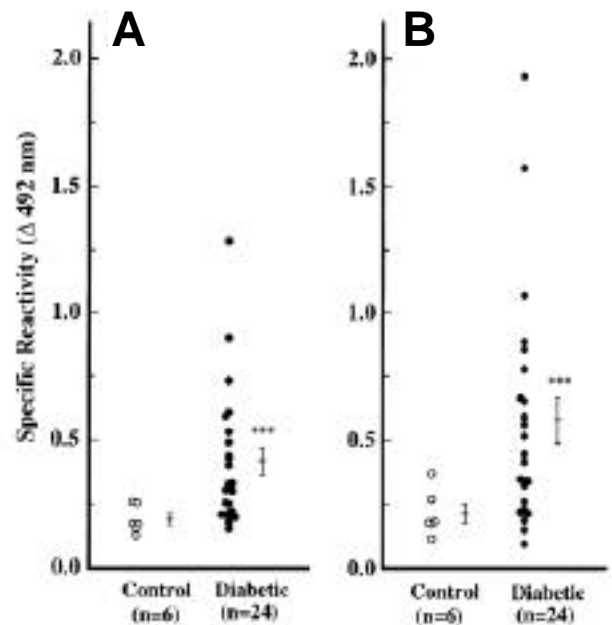


FIG. 3. Specific immunoreactivity of plasma samples of diabetic rats to AGE-BSA and CML-BSA. The reactivity of plasma samples from 24 diabetic rats after 5 weeks of STZ injection (●) and 6 age-matched control rats (○) to AGE-BSA (**A**) and CML-BSA (**B**) was determined by noncompetitive ELISA. The specific reactivity of each plasma sample to AGE-BSA was calculated by subtracting its reactivity to BSA from that to AGE-BSA. Similarly, the specific reactivity of each plasma to CML-BSA was calculated by subtracting its reactivity to BSA from that to CML-BSA and expressed as a difference in absorbance at 492 nm ( $\Delta 492$  nm). Error bars represent SE. \*\*\* $P < 0.001$  vs. age-matched control.

gible (<5%) (data not shown). A similar result was obtained by the competitive ELISA assay; the reactivity of this autoantibody fraction to AGE-BSA (Fig. 4B) or CML-BSA (Fig. 4C) was competitively inhibited by pretreatment with excess AGE-BSA or CML-BSA. Under the same competitive ELISA system, the immunoreactivity of 6D12 toward AGE-BSA and CML-BSA was closely similar to that of the purified autoantibody fraction (data not shown). These results provided convincing evidence for the presence of an autoantibody against CML structure in plasma of diabetic patients.

**Reactivity of the purified human autoantibody fraction to human lens proteins.** Next we examined whether human proteins modified *in vivo* could be in fact recognized by the autoantibody. Because human lens proteins are known to undergo CML modification *in vivo* (9,27,33), we examined the interaction between the purified autoantibody fraction with normal human lens proteins in a noncompetitive ELISA system. The autoantibody did not react in a significant manner with the child's lens (<4 years of age), but showed a positive reaction to adult lenses depending on age; the reactivity to lens tissue from an 89-year-old patient was higher than that to 25-year-old lens (Fig. 5A). The pattern of reaction of the autoantibody to these human lens proteins was similar to that of 6D12 (Fig. 5B). And the reactivity to these human lens proteins was significantly inhibited by the pretreatment with an excess amount of CML-BSA: its reactivity to the 89-year-old lens was inhibited by 64% (Fig. 5A). These findings indicate that CML-modified protein is in fact recognized *in vivo* by the autoantibody in which CML structure plays an important role as an immunological epitope.

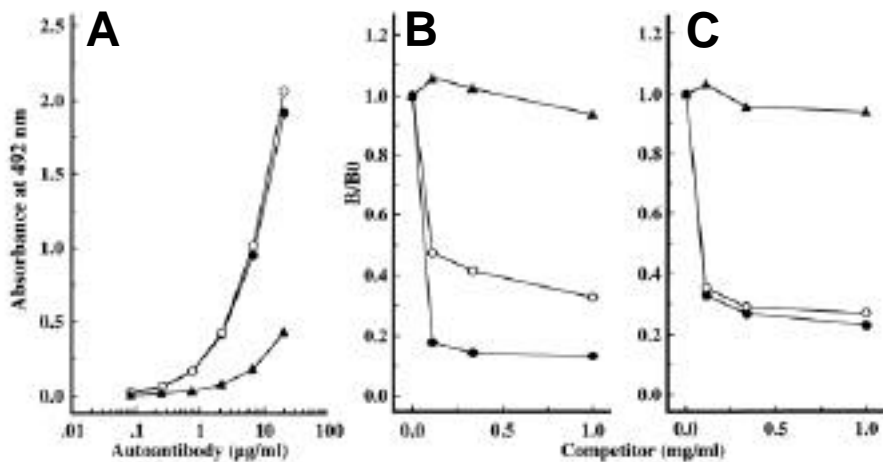


FIG. 4. Autoantibody from plasma of diabetic patients and its immunoreactivity to AGE-BSA and CML-BSA. A: Reactivity of the purified human autoantibody fraction to AGE-BSA (●), CML-BSA (○), and BSA (▲) was determined by non-competitive ELISA. Effects of AGE-BSA, CML-BSA, and BSA on the reactivity of the same human sample to AGE-BSA (B) or CML-BSA (C) was determined by competitive ELISA. Each data point represents the mean of triplicate analyses.

**Human autoantibodies against AGE structure in normal diabetic subjects and nondiabetic patients with renal failure.** To further define the relationship between autoantibody generation and AGE formation in vivo, we compared the autoantibody activity in different diabetic patients with that of normal plasma. For this purpose, human plasma samples were obtained from normal subjects; diabetic subjects without any renal failure, with microproteinuria, with macroproteinuria, and with hemodialysis; and nondiabetic subjects with hemodialysis. From individual plasma samples, the autoantibody fractions reactive to AGE-BSA but not to BSA were partially purified by AGE-BSA- and BSA-conjugated gels. Figure 6 shows the autoantibody activity expressed as specific reactivity, as in Fig. 3. The specific reactivity of the purified antibody fractions from diabetic patients treated by hemodialysis was the highest among the groups. The second and third highest were nondiabetic patients treated by hemodialysis and diabetic patients with macroproteinuria, respectively. The autoantibody activities of the above three groups were statistically higher than normal subjects or diabetic patients without renal failure. Similarly, the specific reactivities of the other two groups (diabetic patients with microproteinuria and diabetic patients without renal failure) were higher than normal subjects, but the difference from normal subjects was not statistically significant. The autoantibody activity in these plasma samples to AGE-BSA was significantly inhibited by CML-BSA, as in the case of Fig. 4B (data not shown).

**DISCUSSION**

Previous studies from our laboratory have shown that AGE proteins such as AGE-BSA prepared in vitro are highly immunogenic for animals such as rabbit and mouse, allowing the development of polyclonal and monoclonal anti-AGE antibodies, respectively (26). Characterization of these polyclonal and monoclonal anti-AGE antibodies suggested the presence of a common epitope on AGE preparations regardless of whether AGE products are generated from proteins, amino acids, or monoaminocarboxylic acids. Subsequent studies by Reddy et al. (33) showed that CML was one of the major immunological epitopes of AGE proteins, and our recent study (27) showed that the epitope of our monoclonal anti-AGE antibody (6D12) was in fact a CML protein adduct. Immunological analyses using 6D12 have demonstrated the presence of AGE-modified proteins in human tissues

(9–13,15,16,19–21,24,25). The presence of AGE proteins in human tissues was also demonstrated immunologically with anti-AGE antibodies whose epitope(s) are unidentified (22,34–36). These anti-AGE antibodies have greatly contributed to our present understanding that human proteins undergo AGE modification during aging and in age-related disease states, implicating the potential presence of autoantibodies against AGE structures in human plasma.

The major finding of the present study was the presence in patients with diabetes or renal failure of an autoantibody against AGE structures, in particular, an autoantibody

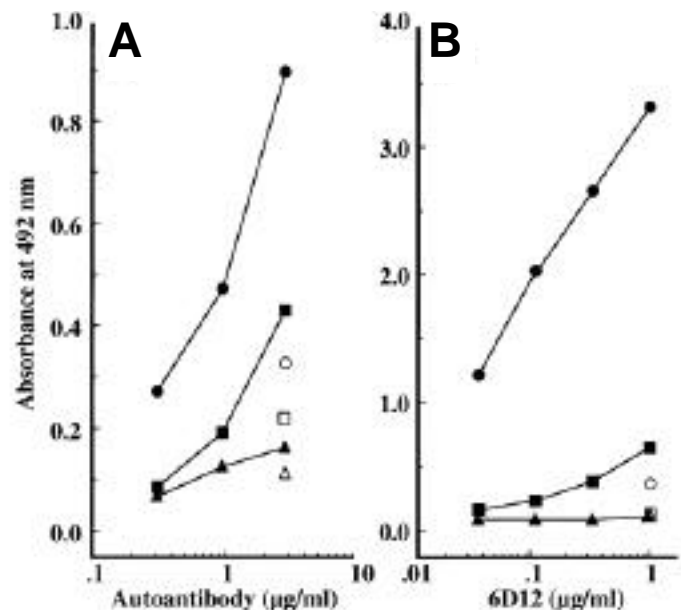
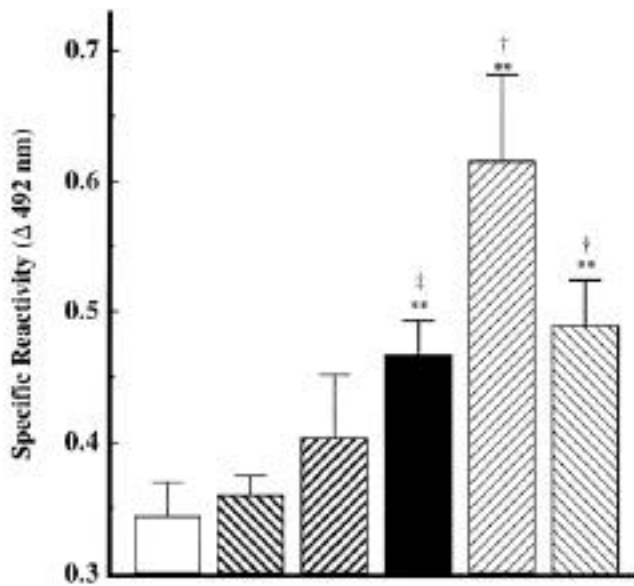


FIG. 5. Immunoreactivity of the purified human autoantibody fraction and 6D12 to human lens proteins. A: Reaction of the purified human autoantibody fraction from diabetic patients toward the water-soluble human lens fractions was determined by noncompetitive ELISA. Three lenses were used: the first from a child <4 years of age (▲), the second from a 25-year-old adult (■), and the third from an 89-year-old elderly subject (●). Effects of CML-BSA on the reactivity of the same human sample to a child <4 years of age (△), a 25-year-old adult (□), and an 89-year-old elderly subject (○) were determined by competitive ELISA. Under the same conditions, the reaction of 6D12 to human lens proteins was determined (B). Each data point represents the mean of triplicate analyses.



**FIG. 6.** Reactivity of purified human autoantibody fractions to AGE-BSA. Plasma samples from normal subjects (□), diabetic patients without renal failure (■), diabetic patients with microproteinuria (▨), diabetic patients with macroproteinuria (■), diabetic patients with hemodialysis (▩), and nondiabetic patients with hemodialysis (■) were used. The specific reactivity of each human autoantibody fraction to AGE-BSA was determined by noncompetitive ELISA. Data are means  $\pm$  SE. \*\* $P < 0.01$  vs. normal group. † $P < 0.05$ , ‡ $P < 0.01$  vs. diabetic patients without renal failure.

against CML structure. This conclusion was supported by three lines of evidence. The first evidence came from animal studies using STZ-induced diabetic rats. In these diabetic animals, the autoantibody activity against AGE-BSA increased with prolongation of diabetic state (Fig. 1). Plasma IgG of diabetic rats reacted not only with AGE-BSA but also with CML-BSA and both of their immunoreactivities were inhibited specifically by CML-BSA, suggesting the presence of an autoantibody against CML structure in plasma of diabetic rats (Figs. 2 and 3). Second, the plasma fraction purified from human diabetic subjects that bound to AGE-BSA but not to BSA showed a positive reaction to both CML-BSA (Fig. 4) and human lens proteins (Fig. 5), which are known to undergo CML modification in vivo (9,27,33). Finally, patients with renal failure caused by diabetes or other etiologies had a higher autoantibody activity against AGE structures, including CML, than normal subjects or diabetic patients without renal failure (Fig. 6). In this regard, it has long been known that the deposition of immunoglobulin and complement occurs in glomeruli of diabetic rats and patients (37–39). Furthermore, it is reported that AGE accumulation in the kidney is more prominent in diabetic nephropathy or chronic renal failure than in normal subjects (11,12). Thus, it is likely that immunocomplexes of autoantibody and AGE accumulated in glomeruli might contribute to the pathogenesis of the above diseases. It remains unclear from these data whether the autoantibody to CML plays some causative or protective role in the pathogenesis of chronic renal failure of diabetic or nondiabetic etiology.

As described above, the presence of autoantibodies against an AGE structure(s) in human plasma has also been expected from the reported fact that AGE-modified proteins are produced in vivo and increase with age. Because AGE

modification is probably very low or negligible during maturation of the immune system, it seems highly reasonable to assume that chemical structure(s) generated on biological proteins because of post-translational modification by glucose could be recognized as a foreign antigen(s) by the immune system, at least in some individuals.

Witztum et al. (40) first reported the presence of autoantibodies against the glycosylated proteins; plasma from diabetic patients could interact with glucitolysine and glucitolysine adducts prepared in vitro by the reduction of Schiff base or Amadori products of glycosylated plasma proteins such as LDL, HDL, and HSA. Furthermore, autoantibodies to oxidized LDL as well as those to malondialdehyde-modified lysine/proteins were detected in sera of Watanabe heritable hypercholesterolemia (WHHL) rabbits, an experimental model of atherosclerosis, and in patients with coronary artery disease (41) as well as accelerated progression of carotid atherosclerosis (42). Results of extensive studies on autoantibodies against oxidized LDL by Palinski and Witztum and their colleagues (43–48) also provided a reasonable basis for in vivo generation of autoantibodies against chemically modified proteins such as oxidized LDL and glucose-modified proteins.

Two reports implicating the potential presence of autoantibodies against AGE proteins are available. First, Palinski et al. (49) demonstrated that advanced atherosclerotic lesions in WHHL rabbits were stained positively by an antibody against 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole (FFI). This finding allowed them to further examine whether the autoantibody against FFI could be present in diabetic patients. Whereas some positive reactions were only found in IgA fractions, no significant reactions were detected in IgG or IgM fractions. FFI was once proposed as an AGE structure but demonstrated later to be an artifactual compound derived from Amadori products (50,51). Based on these reports, the data of Palinski et al. (49) suggest the presence of autoantibodies against FFI-like structures. Second, Baydanoff et al. (52) developed a polyclonal anti-AGE antibody with AGE keyhole limpet hemocyanin in guinea pig according to the method of Nakayama et al. (53) and showed that the reaction of AGE elastin to their anti-AGE antibody was significantly inhibited by serum of diabetic patients. Dorrian et al. (54) reported a similar phenomenon—that the reaction of anti-AGE antibody with AGE proteins or CML proteins was effectively inhibited by human serum but not by heat-treated serum (at 56°C for 30 min). These findings suggest the presence of plasma factors competing for the reaction between anti-AGE antibody and its antigen. It is not clear, however, whether the plasma factor is identical to the autoantibody to AGE proteins. In this sense, to the best of our knowledge, the present study is the first report describing the presence of human autoantibody against CML protein adduct, one of the AGE structures. Recently, Ahmed et al. (55) showed that human lens proteins were modified by N<sup>ε</sup>-(carboxyethyl) lysine (CEL); it is therefore possible that CEL as a CML-like structure also serves as an epitope to raise an autoantibody against AGE structures.

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