N^{ϵ}-(Carboxymethyl)lysine Protein Adduct Is a Major Immunological Epitope in Proteins Modified with Advanced Glycation End Products of the Maillard Reaction†

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Received December 28, 1995; Revised Manuscript Received March 6, 1996⊗

ABSTRACT: Long-term incubation of proteins with glucose leads to the formation of advanced glycation end products (AGE). Recent immunological studies have suggested the potential role of AGE in atherosclerosis, aging, and diabetic complications. We previously prepared a monoclonal (6D12) as well as a polyclonal anti-AGE antibody and proposed the presence of a common AGE structure(s) that may act as a major immunochemical epitope [Horiuchi, S., Araki, N., & Morino, Y. (1991) *J. Biol. Chem. 266*, 7329−7332]. The purpose of the present study was to determine the major epitope. Amino acid analysis of AGE−proteins indicated that N^{ϵ}-(carboxymethyl)lysine (CML) was a major modified lysine residue. Immunologic studies demonstrated the positive reaction of 6D12 not only to all CML-modified proteins tested, but also to BSA modified with several aldehydes known to generate a CML−protein adduct, and a linear correlation between the CML contents of CML-BSA and their immunoreactivity to 6D12 up to ∼8 mol/mol of protein. Further experiments with CML analogs revealed that the epitope of 6D12 is a CML−protein adduct with an important carbonyl group. In contrast to 6D12, our polyclonal anti-AGE antibody showed a significant but much weaker immunoreactivity to CML-BSA, suggesting that the polyclonal antibody contains two populations, one reactive to CML (CML-PA) and the other unreactive to CML (Non-CML-PA). Non-CML-PA separated from CML-PA by CML-BSA affinity chromatography did not react with all CML-modified preparations, but retained its property to react commonly with AGE preparations obtained from proteins, lysine derivatives, and monoaminocarboxylic acids. Therefore, it is clear that a CML−protein adduct is a major immunological epitope in AGE structures, but there still exist other major epitope(s) expressed commonly in AGE−proteins.

Long-term incubation of proteins with glucose leads to the formation of advanced glycation end products (AGE)[1] which are characterized by fluorescence, brown color, and inter- and intramolecular cross-linking (Maillard, 1912; Finot, 1982). Recent studies have suggested the potential role of AGE in atherosclerosis, aging, and diabetic complications (Araki et al., 1992; Makino et al., 1995; Kume et al., 1995). Several AGE structures have been identified including pyrraline (Hayase et al., 1989), pentosidine (Sell et al., 1989), (carboxymethyl)lysine (Ahmed et al., 1986), and crosslines (Nakamura et al., 1992). Immunological studies using antibodies specific for these compounds have confirmed their presence in vivo (Hayase et al., 1989; Ienaga et al., 1995). However, it is still not known whether one of these compounds contributes, as a major AGE structure, to the pathogenesis of these diseases, or whether other structure(s) may involve in this process. Immunological approaches have been attempted to determine the major AGE structure(s) expressed in vivo. Using AGE-BSA as an antigen, we previously prepared a monoclonal anti-AGE antibody (6D12) in mice as well as a polyclonal anti-AGE antibody in rabbits (Horiuchi et al., 1991). Studies examining the immunoreactivity of these antibodies have demonstrated an interesting observation: both antibodies react with AGE samples obtained from proteins, peptides, lysine derivatives, and monoaminocarboxylic acids, suggesting the presence of a common AGE structure(s) in these AGE preparations. Subsequent immunologic studies using 6D12 have disclosed the presence of AGE in several tissues and their potential involvement in disease processes. For instance, the amount of AGE in human lens increased proportionally with the lens age (Araki et al., 1992). AGE were detected in β_2-microglobulin of carpal tunnel amyloid fibril deposits in patients with hemodialysis-associated amyloidosis, suggesting a causative role of AGE modification in the pathogenesis of the disease (Miyata et al., 1993). The presence of AGE has also been demonstrated in renal proximal tubules in patients with diabetic nephropathy and chronic renal failure (Makino et al., 1995; Yamada et al., 1995), in atherosclerotic lesions of arterial walls (Kume et al., 1995), and in brain tissue of Alzheimer's disease (Kimura et al., 1995; Vitek et al., 1994; Smith et al., 1994), pointing to their involvement

† This work was supported in part by Grants-in-Aid for Scientific Research (06454170 and 07557076) from the Ministry of Education, Science and Culture of Japan.

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⊗ Abstract published in *Advance ACS Abstracts,* June 1, 1996.

[1] Abbreviations: AGE, advanced glycation end product(s); BSA, bovine serum albumin; HSA, human serum albumin; Hb, human hemoglobin; CML, N^{ϵ}-(carboxymethyl)lysine; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.

S0006-2960(95)03055-8 CCC: $12.00

in the pathogenesis of diabetic complications, atherosclerosis, and amyloidogenesis of senile plaques.

In spite of these immunological studies, we still do not know the epitope structure recognized by 6D12. Determination of the structure is crucial to the uncovering of the chemical structure of AGE. It is thought that the chemical structure differs from pyrraline and pentosidine since neither of them is recognized by 6D12 (Horiuchi et al., 1991). Since our initial study showed that N^{ϵ}-(carboxymethyl)lysine (CML) did not affect the interaction between 6D12 and AGE-BSA in competitive ELISA, CML as a possible epitope of this antibody has long been neglected. However, the recent report (Reddy et al., 1995) that a CML−protein adduct is a major epitope in polyclonal anti-AGE antibodies stimulated us in the present study to reexamine whether this is the case with our antibodies. The results of the present experiments indicate that the epitope of 6D12 is a CML−protein adduct with a carbonyl group important for immunological recognition of AGE proteins. Furthermore, separation of an antibody population specific for a CML−protein adduct from our polyclonal anti-AGE antibody has clearly showed that a structure(s) other than the CML−protein adduct is also involved as a major epitope.

MATERIALS AND METHODS

Chemicals. BSA (fraction V), HSA (fraction V), human Hb, bovine ribonuclease A (RNase A), methylglyoxal, glycolaldehyde, and DL-glyceraldehyde were purchased from Sigma (St. Louis, MO). Hippuryllysine (benzoylglycyllysine: $M_r = 307$) was obtained from Peptide Institute (Osaka, Japan). D-Glucose, glyoxylic acid, $NaCNBH_3$, and monoiodoacetamide were purchased from Wako Chemical Co. (Osaka). Monoiodoacetic acid, 3-iodopropionic acid, 4-bromobutyric acid, and acetic anhydride were obtained from Aldrich Chemical Co. (Milwaukee, WI). Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG antibody and goat anti-rabbit IgG antibody were purchased from Vector Laboratories (Burlingame, CA). Microtitration plates (96-well; Nunc Immunoplate II) were purchased from Gibco Laboratories (Grand Island, NY). Formylcellulofine gel was from Seikagaku Kogyo Co. (Tokyo, Japan). All other chemicals were of the best grade available from commercial sources.

Preparation of AGE−Proteins. AGE-BSA, AGE-HSA, and AGE-Hb were prepared as described previously (Takata et al., 1988). Briefly, 1.6 g of BSA, 0.5 g of HSA, and 0.25 g of Hb were dissolved with 3.0 g of D-glucose in 10 mL of 0.5 M sodium phosphate buffer (pH 7.4) containing 0.05% NaN_3. Each solution was deoxygenated with nitrogen, sterilized by ultrafiltration (0.45 μm filter), and incubated for 90 days at 37 °C. The samples were then dialyzed against PBS and used for the experiments.

Preparation of Monoclonal Anti-AGE Antibody. The monoclonal anti-AGE antibody was prepared as described previously (Horiuchi et al., 1991). Briefly, splenic lymphocytes from Balb/c mouse immunized with AGE-BSA were fused to myeloma P3U1 cells. The hybrid cells were screened, and two cells lines positive to AGE-HSA but negative to BSA were selected through successive subcloning. One line, termed 6D12, was produced from ascitic fluid of Balb/c mice and further purified by protein A affinity chromatography to IgG_1.

Preparation of Polyclonal Anti-AGE Antibody. The polyclonal anti-AGE antibody was also prepared as described previously by our group (Horiuchi et al., 1991). Briefly, 1.0 mg of AGE-BSA in 50% Freund's complete adjuvant was injected intradermally into each rabbit, followed by five booster injections with the same amount of AGE-BSA. Ten days after intradermal and intramuscular final injection of 2.0 mg of AGE-BSA, serum was taken for further affinity purification. Formylcellulofine gels were coupled to BSA and AGE-BSA as described previously (Horiuchi et al., 1991). The anti-AGE-BSA antiserum (20 mL) was passed twice over a column (1.4 × 10 cm) of formylcellulofine−BSA gel. The nonadsorbed fraction was then passed over a column of formylcellulofine−AGE-BSA gel. The fraction adsorbed to AGE-BSA was eluted with 0.2 M glycine-HCl buffer (pH 2.26). The resultant affinity purified polyclonal anti-AGE-BSA was used for the experiments.

Chemical Modification of Proteins. CML-BSA was prepared as described previously (Ahmed et al., 1986; Dunn et al., 1991). Briefly, 50 mg/mL BSA (45 mM lysine equivalent) was incubated at 37 °C for 24 h with 45 mM glyoxylic acid and 150 mM $NaCNBH_3$ in 10 mL of 0.2 M sodium phosphate buffer (pH 7.4), followed by dialysis against PBS. To prepare CML-BSA samples with different levels of modification, 1.0 mL of BSA solution (50 mg/mL) was incubated in the same way as described above except for various concentrations (from 1.0 to 450 mM) of glyoxylic acid and 5-fold molar ratio of $NaCNBH_3$. Other CML−proteins were also prepared from HSA, human IgG, Hb, and RNase in the same manner. BSA was modified with aliphatic aldehydes as described (Horiuchi et al., 1986). Briefly, BSA (20 mg/mL) was incubated for 5 h at 37 °C with either 50 mM glycolaldehyde, glyceraldehyde, acetaldehyde, formaldehyde, or 150 mM methylglyoxal in the presence or absence of 0.1 M $NaCNBH_3$ in 1.0 mL of 0.1 M sodium carbonate buffer (pH 10.0), followed by overnight dialysis against PBS at 4 °C (Acharya et al., 1983). Modification by acetic anhydride was performed in the same manner except for 0.1 M acetic anhydride (Basu et al., 1976). To prepare other CML analogs, BSA (20 mg/mL) was incubated at 37 °C for 24 h with either 150 mM iodoacetamide, 3-iodopropionic acid, or 4-bromobutyric acid (Heinrikson 1966) in 1.0 mL of 0.1 M sodium carbonate buffer (pH 10.0), followed by overnight dialysis. Protein concentrations were determined by bicinchoninic acid (BCA) protein assay reagent (Pierce) using BSA as a standard (Smith et al., 1985). The extent of chemical modification was determined as described with 2,4,6-trinitrobenzenesulfonic acid as a difference in lysine residues of modified and unmodified protein preparations (Habeeb, 1966). The extent of lysine modification (%) of modified BSA preparations was 87.8% for AGE-BSA, 60.0% for glycolaldehyde-modified BSA, 48.0% for glyceraldehyde-modified BSA, 71.5% for acetaldehyde-modified BSA, 46.6% for formaldehyde-modified BSA, 25.9% for BSA treated with glyoxylic acid and $NaCNBH_3$, 43.2% for BSA treated with methylglyoxal and $NaCNBH_3$, 15.7% for iodoacetamide-modified BSA, 29.2% for BSA treated with glycolaldehyde and $NaCNBH_3$, 18.9% for BSA treated with glyceraldehyde and $NaCNBH_3$, 15.3% for BSA treated with 3-iodopropionic acid, 9.6% for BSA treated with 4-bromobutyric acid, and 58.6% for acetic anhydride-treated BSA. Based on these data, the immunoreactivity of 6D12 to these CML analogs was plotted against moles of modified lysine.

Analytical Procedures. Hippuryl-CML was prepared by incubating 40 mg/mL hippuryllysine with 0.13 M glyoxylic acid in the presence of 0.65 M $NaCNBH_3$ in 0.5 mL of 0.1 M sodium carbonate buffer (pH 10.0) overnight at room temperature (Ahmed et al., 1986; Dunn et al., 1991). CML contents of modified proteins were quantitated by amino acid analysis after hydrolysis of the protein in 6 N HCl for 24 h at 110 °C. Amino acid analysis was performed using an amino acid analyzer (Model 835, Hitachi Co., Tokyo) using an ion exchange HPLC column (2622 SC, 4.6 × 60 mm, Hitachi Co.) and ninhydrin post-column detecting system. The identity of CML detected by HPLC was confirmed by fast atom bombardment (FAB) spectroscopy.

Enzyme-Linked Immunosorbent Assay. Assays were performed at room temperature in two different systems; noncompetitive ELISA and competitive ELISA. In noncompetitive ELISA, each well of a 96-well microtiter plate contained 0.1 mL of the sample to be tested in 50 mM sodium carbonate buffer (pH 9.6) and incubated for 60 min. This was followed by triplicate washing with PBS containing 0.05% Tween 20 (buffer A). Each well was blocked with 0.5% gelatin, washed with buffer A, and reacted for 60 min with 0.1 mL of buffer A containing 0.01 μg of the monoclonal (6D12) or the polyclonal anti-AGE antibody. The wells were then washed with buffer A and reacted with HRP-conjugated anti-mouse IgG (for monoclonal anti-AGE-BSA) or HRP-conjugated anti-rabbit IgG (for polyclonal anti-AGE-BSA), followed by reaction with 1,2-phenylenediamine dihydrochloride. The reaction was terminated by 1.0 M sulfuric acid, and the absorbance at 492 nm was read on a micro-ELISA plate reader. In competitive ELISA, each well was coated with 0.1 mL of 1.0 μg/mL of AGE-BSA, blocked with 0.5% gelatin, and washed three times with buffer A. The sample to be tested (60 μL) was mixed with equal volume or buffer A containing 0.01 μg of 6D12 or the polyclonal anti-AGE antibody and incubated for 60 min. A portion of the mixture (100 μL) was added to each well of a 96-well microtiter plate prepared above, followed by 60 min incubation. The wells were then washed three times with buffer A, and the antibodies bound to wells were dected by HRP-conjugated anti-mouse or anti-rabbit IgG in a manner similar to that described above for the noncompetitive ELISA (Horiuchi et al., 1991). For experiments using ELISA, the same set of experiment was repeated at least three times and the representative data were shown in each figure.

Separation of CML-Specific Antibody from Polyclonal Anti-AGE Antibody by CML-BSA Affinity Chromatography. CML-BSA was coupled to formylcellulofine gel as described previously by our laboratory (Ohta et al., 1989). Briefly, 8 mg of CML-BSA was reincubated for 3 h at room temperature with 3 g of formylcellulofine gel in 4 mL of 0.1 M sodium phosphate buffer (pH 7.4) containing 0.2 M NaCl and 0.05% NaN_3. $NaCNBH_3$ (28 mg) was added to the solution, and the mixture was incubated overnight at room temperature. The unreacted formyl groups of the gel were blocked by 2 h incubation with 1.0 M monoethanolamine and 50 mM $NaCNBH_3$. The gel was washed, equilibrated with PBS, and packed to the column (1 × 4.3 cm). About 93% of CML-BSA was bound to the formylcellulofine gel. The polyclonal anti-AGE-BSA antibody (10.8 mg in 1.5 mL of PBS) was loaded onto the column. The column was washed with 40 mL of PBS at a flow rate of 9 mL/h to obtain the nonadsorbed fraction. The adsorbed fraction was then eluted with 30 mL of 0.2 M glycine-HCl buffer (pH 2.26), and the eluate was neutralized with 1.0 M tris(hydroxymethyl)aminomethane. Each fraction (1.0 mL) was monitored for absorbance at 280 nm. Both the adsorbed and nonadsorbed fractions were pooled, concentrated with Centriprep-10 (Amicon), dialyzed against PBS, and used for the study. The immunoreactivity of each fraction to AGE−proteins and CML derivatives was determined by noncompetitive and competitive ELISA as described above.

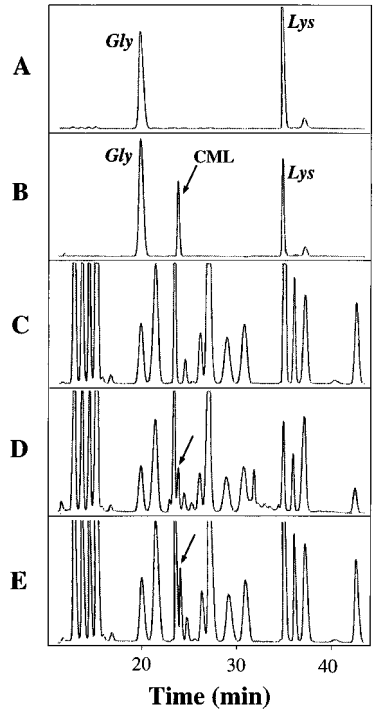

FIGURE 1: Identification of CML in AGE-BSA by amino acid analysis. CML contents of modified proteins were measured by amino analysis as described in Materials and Methods. Glycine (Gly) and lysine (Lys) in acid hydrolysates of hippuryllysine were eluted at 20.22 and 36.08 min, respectively (A). In the case of hippuryl-CML, the peak corresponding to lysine was reduced and a new peak (arrow) appeared with a retention time of 24.37 min, while the peak corresponding to glycine did not change (B). Whereas acid hydrolysates obtained from BSA showed no significant peak other than that of native amino acids (C), those obtained from AGE-BSA gave a new peak (arrow) with a retention time (24.37 mm) identical to that of CML detected in hippuryl-CML (D). Other new peaks were also found in acid hydrolysates of AGE-BSA (D). From acid hydrolysates of CML-BSA, only one peak corresponding to CML was detected (arrow) (E).

RESULTS

Identification of CML in AGE Preparations. The detection system for CML was established with an HPLC system in which glycine and lysine obtained by acid hydrolysis of hippuryllysine (Bz-glycyllysine) were eluted in two peaks at 20.22 and 36.08 min, respectively (Figure 1A). Hippuryllysine was carboxymethylated with glyoxylic acid in the presence of $NaCNBH_3$ and subjected to acid hydrolysis, followed by HPLC analysis. The peak, corresponding to lysine, decreased in intensity, but a new peak emerged at a retention time of 24.37 min, while the peak corresponding to glycine did not change, clearly indicating that the peak eluted at 24.37 min is CML (Figure 1B). The latter peak was detected in acid hydrolysates obtained from AGE-BSA (Figure 1D) and CML-BSA (Figure 1E), but not in BSA

Table 1: CML Contents of Modified Proteins and Their Immunoreactivity to 6D12

sample	CML content[a] mmol/mol of lysine	CML content[a] mol/mol of protein	concns required for 50% inhibition (nM)
AGE-BSA	130	7.69	38
CML-BSA	157	9.25	50
CML-HSA	99	5.84	26
CML-Hb	49	2.14	86
glyceraldehyde-BSA	15	0.91	46
glycolaldehyde-BSA	30	1.78	79
formaldehyde-BSA	ND[b]	ND	
acetaldehyde-BSA	ND	ND	
hippuryl-CML	314		9016

[a] The CML content of each modified product was quantitated by amino acid analysis as described under Materials and Methods and was expressed mmol/mol of lysine or mol/mol of protein or both. The immunoreactivity of each modified product to 6D12 was determined by competitive ELISA as described under Materials and Methods. The extents of their immunoreactivity shown in Figures 2−4 were expressed as concentrations required for 50% inhibition. [b] ND, not detected.

(Figure 1C). Using this method, we determined CML contents of our AGE preparations used for the present study (Table 1).

Immunological Reactivity of 6D12 to CML−Proteins and Hippuryl-CML. To determine whether the CML−protein is an eiptope of our monoclonal antibody (6D12), we examined the immunological reaction of 6D12 with CML−proteins. In noncompetitive ELISA (Figure 2A), CML-BSA reacted with the antibody as effectively as AGE-BSA did. The antibody also recognized other CML−proteins such as CML-HSA, CML-IgG, CML-Hb, and CML-RNase, whereas the unmodified proteins were not recognized by the antibody. A similar result was obtained by the competitive ELISA, although the reactivity of CML-IgG and CML-Hb was slightly weaker than the others (Figure 2B). These results suggest that the CML moiety of AGE-BSA might be an epitope of 6D12. To test this, the antibody was further characterized by its reactivity toward a CML−peptide. As shown in Figure 3, the reactivity of 6D12 to AGE-BSA was significantly inhibited by hippuryl-CML. At a CML equivalent basis, however, its inhibitory effect was much weaker: 5.6% of CML-BSA (16.77 mol of CML/mol of protein), 10.0% of CML-BSA (6.26 mol/mol), 12.2% of CML-BSA (4.78 mol/mol), and 15.6% of CML-BSA (2.87 mol/mol), suggesting that a CML−protein adduct (CML-like structure) is recognized much more favorably than a CML−peptide.

Immunological Reactivity of 6D12 to Aldehyde-Modified Proteins. Since protein modification with aliphatic aldehydes is known to generate CML−protein adducts (Glomb & Monnier, 1995), we next examined the immunoreaction of 6D12 with aldehyde-modified BSA preparations. The CML contents measured by amino acid analysis were 1.78 mol/mol of protein for glycolaldehyde-treated BSA and 0.91 mol/mol of protein for glyceraldehyde-treated BSA. However, CML was not detectable in formaldehyde-treated BSA nor acetaldehyde-treated BSA. Results of the noncompetitive ELISA (Figure 4A) showed that glycolaldehyde-treated and glyceraldehyde-treated and acetaldehyde-treated BSA. A similar result was obtained in experiments with the competitive ELISA (Figure 4B). Since it is known that CML-protein adducts are generated by interaction with the former two aldehydes, but not with the latter two aldehydes, these data

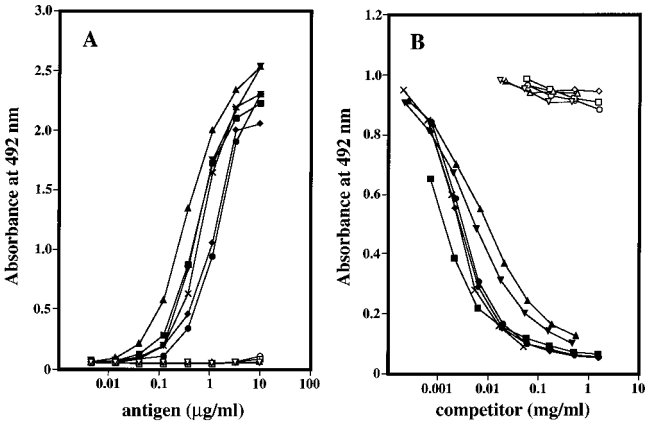

FIGURE 2: Immunoreactivity of monoclonal anti-AGE antibody (6D12) to CML−proteins. (A) Noncompetitive ELISA. Each well of a 96-well microtiter plate was incubated for 60 min with 0.1 mL of the test sample in 50 mM sodium carbonate buffer (pH 9.6) and washed three times with PBS containing 0.05% Tween 20 (buffer A). Each well was blocked with 0.5% gelatin, washed with buffer A, and reacted for 60 min with 0.1 mL of buffer A containing 0.01 μg of 6D12. Wells were then washed with buffer A and reacted with HRP-conjugated anti-mouse IgG followed by reaction with 1,2-phenylenediamine dihydrochloride. The tested samples were AGE-BSA (×), CML-BSA (●), CML-HSA (■), CML-IgG (▲), CML-Hb (▼), and CML-RNase (♦). BSA (○), HSA (□), IgG (△), HB (▽), and RNase (◊) incubated in parallel without glyoxylic acid were also tested as control. (B) Competitive ELISA. Each well was coated with 0.1 mL of 1.0 μg/mL AGE-BSA, blocked with 0.5% gelatin, and washed three times with buffer A. The test sample (60 μL) was mixed with 60 μL of buffer A containing 0.01 μg of 6D12 and incubated for 60 min. A portion of the mixture (100 mL) was then added to each well of a 96-well microtiter plate prepared above, followed by incubation for 60 min. The wells were then washed three times with buffer A, and the antibodies bound to wells were detected by HRP-conjugated anti-mouse IgG in the same way as described above for the noncompetitive ELISA. The tested samples were the same as those in panel A. Experimental details are described under Materials and Methods.

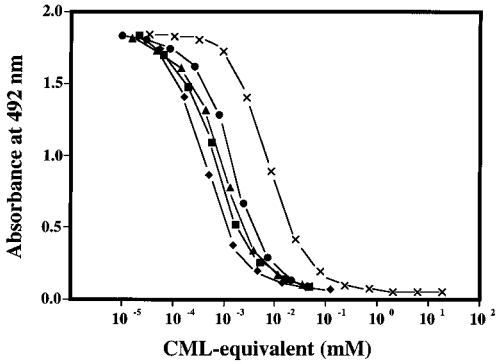

FIGURE 3: Immunoreactivity of 6D12 to CML-BSA and hippuryl-CML. Assays were performed by the competitive ELISA in the same way as in Figure 2B. The tested samples were CML-BSA with different extents of modification: 2.87 (●), 4.78 (▲), 6.26 (■), and 16.77 (♦) mol of CML/mol of protein, and hippuryl-CML (×). The concentration of each CML sample is expressed as CML equivalent, measured by amino acid analysis.

support the notion that a CML−protein adduct is the epitope of 6D12. Furthermore, when the CML contents of modified proteins, such as AGE−proteins, CML−proteins, and aldehyde-modified proteins, were compared with their immunoreactivity to 6D12 (Table 1), one can draw a strict rule that modified proteins without CML−protein adducts cannot be recognized as antigens.

Correlation of CML Contents with Immunological Recognition by 6D12. In order to determine the stoichiometric

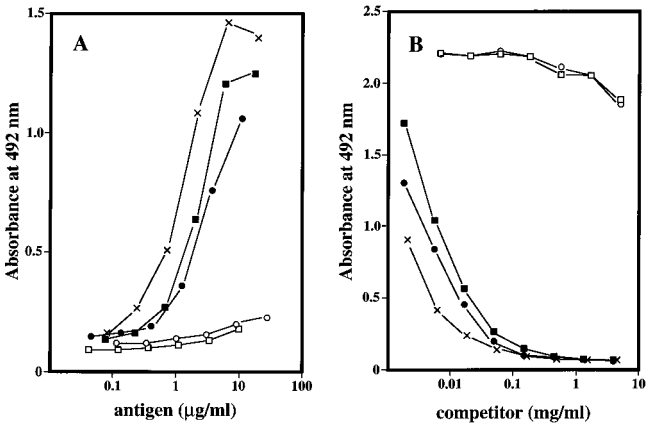

FIGURE 4: Immunoreactivity of 6D12 to aldehyde-modified proteins. The immunoreactivity of 6D12 to various concentrations of glyceraldehyde-modified (●), glycolaldehyde-modified (■), formaldehyde-modified (○), and acetaldehyde-modified BSA (□) was determined by noncompetitive ELISA (A) and competitive ELISA (B). The immunoreactivity to AGE-BSA was determined in parallel as a control (×). See Materials and Methods for experimental details.

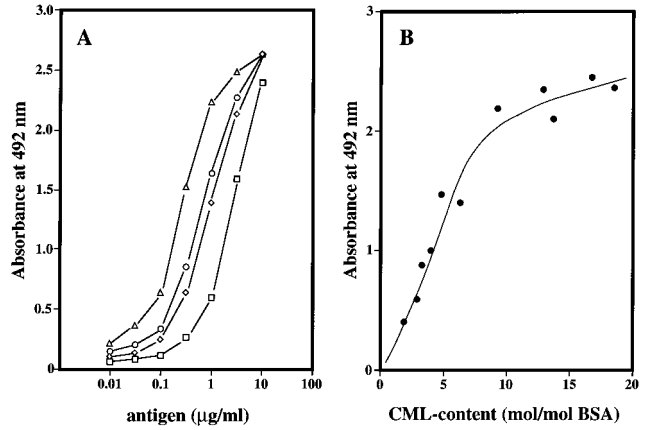

FIGURE 5: Correlation of CML contents with immunological recognition by 6D12. CML-BSA samples with different extents of modification were prepared: 2.87 (□), 4.78 (◇), 6.26 (○), and 16.77 (△) mol of CML/mol of protein, and their immunoreactivities to 6D12 were determined by the noncompetitive ELISA (A). The immunoreactivity of each sample at a fixed concentration (1.0 μg/mL) as shown by absorbance at 492 nm was plotted against CML contents of each sample (B).

correlation between the CML contents of proteins and their immunoreactivity to 6D12, CML-BSA samples with different levels of modification were prepared and their immunoreactivity to 6D12 was determined by noncompetitive ELISA. The immunoreactivity of these CML-BSA samples appeared to increase with increments in CML contents of these samples (Figure 5A). When their immunoreactivities determined at 1.0 μg/mL were plotted against the CML content of these samples, the immunoreactivity increased linearly up to approximately 8 mol/mol of protein and appeared to reach a plateau at levels >10 mol/mol of protein (Figure 5B). Thus, it is likely that the recognition of CML-BSA increased with increasing content of CML, reaching a maximal response at ~8−10 mol/mol of protein.

Immunological Reactivity of 6D12 to CML Analogs. To further characterize the epitope structure recognized by 6D12, CML analogs were prepared by treating BSA with various aldehydes in combination with $NaCNBH_3$. The reactivity of these CML analogs determined by the competitive assay and their expected chemical structures of modified lysine residues are summarized in Table 2. BSA was treated with methylglyoxal and $NaCNBH_3$ to obtain compound **b** (N^{ϵ}-acetonyllysine) in which a carboxylate anion of CML was converted to a ketone, thus removing an anionic charge. BSA was also modified by iodoacetamide to replace the hydroxyl group of CML by an amino group [compound **c**: N^{ϵ}-(carbamoylmethyl)lysine]. The immunoreactivity of 6D12 to compounds **b** and **c** was still significant but much weaker than its reactivity to the authentic CML−protein adduct (compound **a**: CML) by 0.6% and 0.3%, respectively. In contrast, when BSA was modified by both glycolaldehyde and $NaCNBH_3$ to obtain compound **d** [N^{ϵ}-(hydroxyethyl)-lysine], its immunoreactivity to the antibody totally disappeared. When BSA was modified with glyceraldehyde in the presence of $NaCNBH_3$ to obtain compound **e** [N^{ϵ}-(2,3-dihydroxypropyl)lysine], 6D12 did not show any reactivity to this compound. These data indicate that the carbonyl group of C-1 position in the CML structure is essential while the hydroxyl group of CML is less important. Furthermore, replacement of the carboxymethyl group of CML with a carboxyethyl group by modification with 3-iodopropionic acid [compound **f**: N^{ϵ}-(carboxyethyl)lysine)] resulted in a marked reduction (1.9%) of its immunoreactivity to 6D12. One more methyl group was added to compound **f** by treating with 4-bromobutyric acid to form compound **g** [N^{ϵ}-(carboxypropyl)lysine]. Furthermore, the methyl group was deleted from CML by modification with acetic anhydride to form compound **h** (N^{ϵ}-acetyllysine). However, both compounds **g** and **h** were no longer recognized by 6D12. Thus, it is likely that the methyl group of CML is also important. Based on these results, it is evident that 6D12 is quite specific for CML structure of a CML−protein adduct.

Presence of a Major Immunological Epitope(s) Other Than CML−Protein Adducts in Polyclonal Anti-AGE Antibody. Our previous study showed the presence of a common epitope recognized by both the polyclonal and monoclonal anti-AGE antibody (Horiuchi et al., 1991). Since the above data identified the CML−protein adduct as an epitope of 6D12, the common epitope would be CML. To find out whether CML could also be a major epitope in our polyclonal anti-AGE antibody, we examined its immunoreactivity to CML-BSA. As shown in Figure 6, our antibody did react with CML-BSA, buth much weaker compared with AGE-BSA (Figure 6A). When compared with the competitive ELISA, its reactivity to CML-BSA was significantly weaker than that to AGE-BSA (Figure 6B). It is, therefore, likely that an antibody population specific for CML-protein adducts is present, but other antibodies also exist in our polyclonal anti-AGE antibody. To assess the amount of CML-specific antibody in the polyclonal antibody, CML-specific antibody populations were separated from those unreactive to CML by a CML-BSA affinity chromatography. As shown in Figure 7, about 60% of the polyclonal antibody applied to the column did not adsorb to the affinity gel and eluted as a first peak as "nonadsorbed fraction", whereas about 30% of the antibody adsorbed to the gel and was eluded in a second peak as "adsorbed fraction". As expected, the adsorbed fraction showed a positive immunoreaction toward CML−proteins, such as CML-BSA, CML-HSA, CML-IgG, CML-Hb, and CML-RNase (Figure 8), whereas the nonadsorbed fraction did not react at all with them (Figure 9). These data indicate that 30% of our polyclonal antibody is explained

Table 2: CML Analogs and Their Immunoreactivity to 6D12[a]

name	structure	concn at 50% inhibition	crossreactivity
a: CML	BSA-$NHCH_2C(=O)OH$	0.46	100
b: N^{ϵ}-acetonyllysine	BSA-$NHCH_2C(=O)CH_3$	76	0.6
c: N^{ϵ}-(carbamoylmethyl)lysine	BSA-$NHCH_2C(=O)NH_2$	153	0.3
d: N^{ϵ}-(hydroxyethyl)lysine	BSA-$NHCH_2CH_2OH$		ND[b]
e: N^{ϵ}-(2,3-dihydroxypropyl)lysine	BSA-$NHCH_2CH(OH)CH_2OH$		ND[b]
f: N^{ϵ}-(carboxyethyl)lysine	BSA-$NHCH_2CH_2C(=O)OH$	25	1.9
g: N^{ϵ}-(carboxypropyl)lysine	BSA-$NHCH_2CH_2CH_2C(=O)OH$		ND[b]
h: N^{ϵ}-acetyllysine	BSA-$NHC(=O)CH_3$		ND[b]

[a] CML analogs were prepared by treating BSA with various aldehydes in combination with $NaCNBH_3$ as described under Materials and Methods. The immunoreactivity of each analog was determined by competitive ELISA as described under Materials and Methods. The crossreactivity of each CML analog was expressed with the immunoreactivity of CML defined as 100%. [b] ND, not detected.

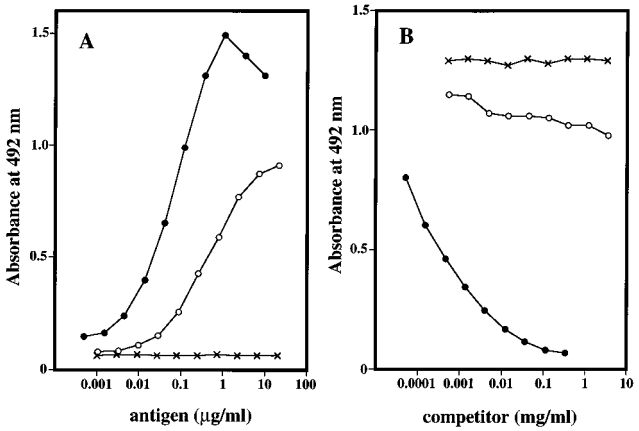

FIGURE 6: Immunoreactivity of polyclonal anti-AGE antibody to CML-BSA. The immunoreactivity of the polyclonal anti-AGE antibody to AGE-BSA (●), CML-BSA (○), and native BSA (×) was determined at various concentrations by noncompetitive ELISA (A) and competitive ELISA (B). Experimental details are described under Materials and Methods.

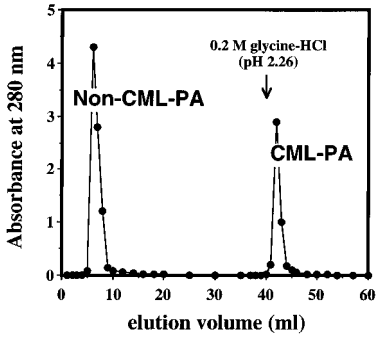

FIGURE 7: Separation of CML-specific Antibody from polyclonal anti-AGE Antibody by CML-BSA affinity chromatography. The polyclonal anti-AGE antibody was loaded onto a CML-BSA column (1 × 4.3 cm), washed with 40 mL of PBS to obtain the nonadsorbed fraction (Non-CML-PA), and then eluted with 30 mL of 0.2 M glycine-HCl buffer (pH 2.26) to obtain the adsorbed fraction (CML-PA). Each fraction (1.0 mL) was monitored for absorbance at 280 nm. Both the nonadsorbed and adsorbed fractions were pooled, concentrated, and dialyzed against PBS, as described under Materials and Methods.

by those reactive to CML−protein adducts (CML-PA) and 60% by those unreactive to CML−protein adducts (Non-CML-PA).

We next characterized the immunoreactivity of the nonadsorbed fraction (Non-CML-PA). As shown in Figure 9, Non-CML-PA reacted positively not only with AGE−proteins, such as AGE-BSA, AGE-HSA, AGE-Hb, AGE-RNase, and AGE-collagen (Figure 9A), but also with AGE preparations obtained from α-tosyl-L-lysine methyl ester and

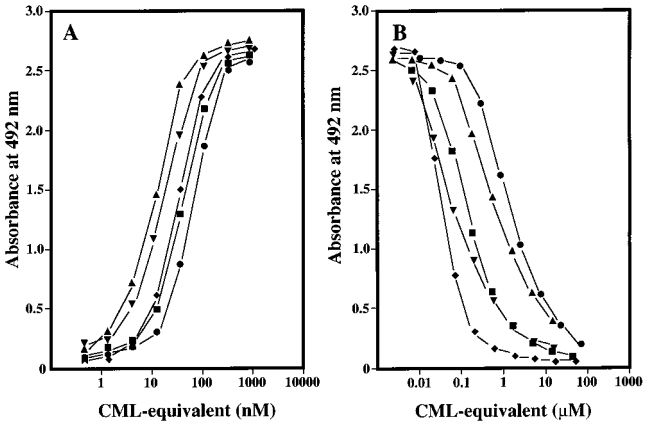

FIGURE 8: Immunoreactivity of the adsorbed fraction (CML-PA) to CML−proteins and AGE-BSA. The immunoreactivity of the adsorbed fraction (CML-PA) to CML−proteins such as CML-BSA (●), CML-HSA (■), CML-IgG (▲), CML-Hb (▼), and CML-RNase (♦) was determined by noncompetitive ELISA (A) and competitive ELISA (B) as described under Materials and Methods and was plotted against CML equivalent.

monoaminocarboxylic acids (Figure 9B), suggesting that Non-CML-PA still recognizes the epitope(s) which is not CML but is commonly expressed by AGE preparations (Non-CML).

Immunological Detection of CML and Non-CML during Maillard Reaction. To confirm the formation of CML during the Maillard reaction and its correlation with that of Non-CML, the amount of CML and Non-CML was immunologically quantitated by the noncompetitive ELISA using the two antibodies obtained above. Each tube containing 50 mg/mL BSA was incubated with 2.0 M glucose at 37 °C for 1−62 days in 1.0 mL of 0.5 M sodium phosphate buffer (pH 7.4). The formation of CML became detectable on day 1, and the level increased rapidly up to day 5. With continued incubation, the CML level slowly but progressively increased to day 62, exhibiting a saturating tendency (Figure 10). In contrast to CML, Non-CML was not detected before day 5, but became detectable on day 13 and increased proportionately with time.

DISCUSSION

Our results clearly demonstrate that the epitope of 6D12 is a CML−protein adduct with a carbonyl group important for immunological recognition of AGE−proteins. Furthermore, experiments with the polyclonal anti-AGE antibody established that a structure(s) other than the CML−protein adduct is also involved as a major immunological epitope among AGE structures.

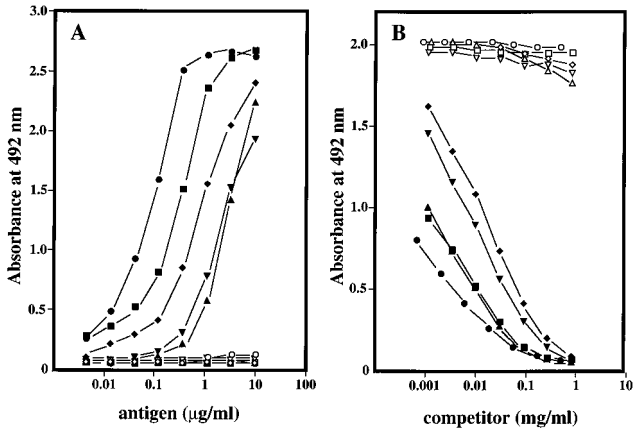

FIGURE 9: Immunoreactivity of the nonadsorbed fraction (Non-CML-PA) to various AGE preparations. The immunoreactivity of the nonadsorbed fraction (Non-CML-PA) to AGE−proteins and CML−proteins was determined by noncompetitive ELISA. Tested samples included AGE-BSA (●), AGE-HSA (■), AGE-RNase (▲), AGE-collagen (▼), AGE-Hb (♦), CML-BSA (○), CML-HSA (□), CML-RNase (△), CML-collagen (▽), and CML-Hb (◇) (A). The immunoreactivity of Non-CML-PA to various concentrations of AGE-lysine derivatives and AGE-monoaminocarboxylic acids was determined by competitive ELISA (B). The tested samples were AGE-BSA (●), CML-BSA (○), AGE-α-tosyl-L-lysine methyl ester (■), α-tosyl-L-lysine methyl ester (box), AGE-ϵ-aminocaproic acid (▲), ϵ-aminocaproic acid (△), AGE-γ-amino-*n*-butyric acid (▼), γ-amino-*n*-butyric acid (▽), AGE-β-alanine (♦), and β-alanine (◇). Experimental details are described under Materials and Methods.

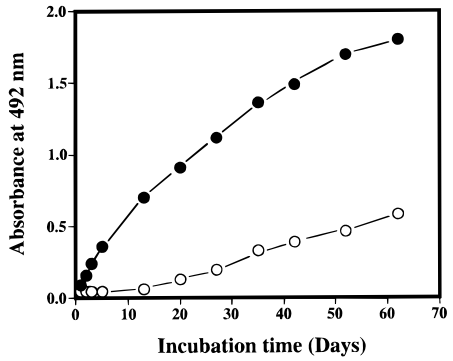

FIGURE 10: Kinetics of CML and Non-CML formation in the Maillard reaction. Each tube contained 50 mg/mL BSA and 2.0 M glucose in 1.0 mL of 0.5 M sodium phosphate buffer (pH 7.4), followed by incubation at 37 °C for 1−62 days. The amount of CML and Non-CML was quantitated by the noncompetitive ELISA using CML-PA (●) and Non-CML-PA (○). The immunoreactivity of each sample to these antibodies was determined at a fixed concentration (0.1 μg/mL). Under these conditions, the reactivities of CML-PA and Non-CML-PA to 0.1 μg/mL AGE-BSA showed 1.703 and 1.748 in absorbance at 492 nm, respectively.

CML−Protein Adducts as a Major Epitope of 6D12. Until the present results became available, we assumed that CML of AGE−proteins does not play an important role as an epitope of 6D12 for two reasons. First, CML is released intact during acid hydrolysis. However, we could not detect its epitope activity by a competitive ELISA after acid hydrolysis of AGE−proteins (Araki et al., 1992). Second, more directly, we failed to observe a significant inhibitory effect of the synthesized CML on the interaction between 6D12 with AGE−proteins under the same ELISA system. Some of these observations may be explained by the present finding that the immunoreactivity of 6D12 to hippuryl-CML, a carboxymethylated low-molecular-weight compound, was significantly weaker than CML-BSA (Figure 3). The precise reason why the immunoreactivity of CML−proteins is stronger than that of the small-sized CML−peptide is not clear. Since the recognition of CML-BSA increased with increasing contents of CML, reaching a maximal response at ∼8−10 mol/mol of protein (Figure 5B), it is probably related to the number of CML incorporated into the modified proteins, as suggested by the previously study (Reddy et al., 1995). It is also possible, however, that the epitope of 6D12 is not restricted only to the CML moiety, but extends to neighboring peptides from the modified lysine residue (i.e., "CML−protein adduct" as it is termed in the present study).

Immunocytochemical studies using 6D12 have demonstrated the presence of AGE in various tissues including the lens (Araki et al., 1992), carpal tunnel amyloid fibril deposits from patients with hemodialysis-associated amyloidosis (Miyata et al., 1993), renal proximal tubules from patients with diabetic nephropathy and chronic renal failure (Makino et al., 1995; Yamada et al., 1995), brain tissue from patients with Alzheimer's disease (Kimura et al., 1995), and aortic atherosclerotic lesions (Kume et al., 1995). Since it is now clear that the CML−protein adduct is the epitope of 6D12, the positive reactions observed in these immunohistochemical studies are derived from CML−protein adducts generated in situ. On the other hand, the in vivo presence of AGE was also demonstrated by other immunological studies using polyclonal anti-AGE antibodies whose precise epitope structure(s) were not known (Nakayama et al., 1993; Mitsuhashi et al., 1993) and plasma (Makita et al., 1992a; Papanastasiou et al., 1994). Particularly, ELISA systems established by the polyclonal antibody raised against AGE-RNase have successfully quantitated levels of AGE in plasma (Makita et al., 1992a), hemoglobin (Makita et al., 1992b), and low-density lipoprotein (Bucala et al., 1993). It is of utmost importance to determine the epitope(s) of these antibodies. Based on the present and previous findings (Reddy et al., 1995) and a CML−protein adduct is one of major epitopes in AGE structures, it is possible in these studies that the compounds detected by their polyclonal antibodies may reflect, in part, CML−protein adducts formed on these tissue and plasma proteins. In fact, the anti-AGE antibody prepared in guinea pigs against AGE-keyhold limpet hemocyanin (KLH) (Nakayama et al., 1993; Mitsuhashi et al., 1993) reacted with CML-BSA as effectively as AGE-BSA (H. Nakayama et al., personal communications). Therefore, reexamination of the immunoreactivity of the polyclonal antibodies to CML−protein adducts is necessary in order to determine the AGE-positive material in tissues and plasma detected by these antibodies.

CML among AGE Structures. The main pathway for CML formation is ascribed to the oxidative cleavage of the Amadori product (Ahmed et al., 1986; Dunn et al., 1991). The recent study (Glomb & Monnier, 1995) suggested that glyoxal/glycolaldehyde derived from Schiff base adduct via the "Namiki pathway" (Hayashi & Namiki, 1986) may also play an important role in CML formation in vivo. It is likely, therefore, that CML is formed at a much earlier stage than the formation of Non-CML-AGE in the Maillard reaction. In our in vitro incubation of BSA with glucose, the amounts of CML and Non-CML were determined immunochemically using CML-PA and Non-CML-PA, respectively (Figure 10). The formation of CML was positively detected on day 1, and its amount increased proportionately with time up to day 5, followed by a gradual increase thereafter with a saturable

tendency. In contrast, Non-CML structure(s) among AGE could not be detected before day 5. On day 13, it became detectable at a significant level and its amount increased thereafter with incubation time up to day 62 under our in vitro system (Figure 10). Thus, it is evident that CML formation starts much earlier than the formation of other AGE structures.

In the previous experiment (Reddy et al., 1995) the interaction of the rabbit polyclonal anti-AGE-KLH antibody with AGE-rabbit serum albumin was completely inhibited by CML-BSA, whereas the inhibitory effect of CML-BSA on the interaction of our rabbit polyclonal anti-AGE antibody with AGE-BSA was partial (about 23%, Figure 6B). The difference in reactivity to CML-BSA in these two studies may probably be derived from the relative ratio of CML contents to Non-CML contents in the AGE−proteins used for the immunogen. Our preliminary experiments with different batches of AGE-BSA preparations showed that the ratio is higher in AGE-BSA preparations obtained by a short-term incubation compared with those obtained by a long-term incubation. This notion is strongly supported by the present result obtained by CML-PA and Non-CML-PA (Figure 10).

Although accumulation of CML-modified proteins in vivo is well documented in several human and animal tissues and is enhanced during normal aging as well as in certain pathological conditions (Dunn et al., 1989, 1991; Knecht et al., 1991; Dyer et al., 1993), the pathophysiological significance of the CML modification is not known. AGE−proteins are believed to elicit several biological phenomena via the AGE-binding protein or the AGE receptor (Takata et al., 1988; Yang et al., 1991; Schmidt et al., 1992; Araki et al., 1995). Previous studies have disclosed that the increase in net negative charge, a property commonly observed among AGE−proteins, plays an important role in the recognition by the AGE receptor (Araki et al., 1995; Vlassara et al., 1986). Since CML modification of proteins increases the net negative charge of proteins, this could contribute to some extent of the AGE receptor-mediated recognition. Further studies are needed to resolve this tissue.

Why do CML−protein adducts have a strong immunogeneicity among a number of AGE structures? Several reasons are available. First, on the quantitative basis, a CML−protein adduct is dominant among modified lysine residues, thus acting as a major antigen in the immune system. In fact, CML explained at least 14.8% of modified lysine residues in our AGE-BSA preparation. Second, CML−protein adducts are formed on the surface lysine residues of the proteins which are susceptible to oxidation in vivo, thus readily undergoing CML modification. Finally, the antigenicity of a CML−protein adduct could be stronger, by yet unknown mechanisms, than that of other AGE structures.

Epitope(s) Other Than CML−Protein Adducts. Since the immunoreactivity of our polyclonal anti-AGE antibody to CML-BSA was much weaker than that to AGE-BSA and its immunoreactivity to AGE-BSA was only partially (23%) competed for by excess CML-BSA (see Figure 6B), it is suggested that our polyclonal antibody contains at least two populations, one reactive to CML (CML-PA) and the other unreactive to CML (Non-CML-PA). This suggestion was demonstrated to be the case by the separation of these two antibody populations from the polyclonal antibody by a CML-BSA affinity column chromatography (Figure 7). As expected, CML-PA reacted with CML−proteins (Figure 8), while Non-CML-PA did not (Figure 9). However, Non-CML-PA still retained its capacity to react with AGE preparations obtained either from proteins, lysine derivatives, or monoaminocarboxylic acids, indicating that Non-CML-PA recognizes a common epitope of AGE structures (Figure 9). It is likely, therefore, that approximately 30% of our polyclonal antibody recognizes a CML−protein adduct while the other 60% recognizes an AGE structure(s) common but different from CML. The epitope structure of Non-CML-PA seems to differ from Amadori products or AGE structures such as pyrraline and pentosidine because neither of them was recognized by our polyclonal antibody (Horiuchi et al., 1991) and because BSA preparations conjugated with pyrraline and pentosidine were not recognized by Non-CML-PA (data not shown). As was suggested by Reddy et al. (1995), determination of an AGE structure(s) other than CML is valuable in identifying the new AGE products. In fact, we recently obtained monoclonal antibodies which did not react with CML−proteins but recognized AGE−proteins.[2] Experiments using these antibodies are in progress to determine whether AGE structures other than CML exist in vivo.

[2] K. Ikeda et al., unpublished observations.

ACKNOWLEDGMENT

We are grateful to Dr. Norie Araki, Ryoji Nagai, and Tetsushi Saishoji of our laboratory for collaborative endeavors and helpful discussion during the course of this study, and to Tamami Sakamoto of Panapharm Laboratories (Kumamoto, Japan) for the preparation of 6D12. We also thank Dr. F. G. Issa from the Department of Medicine, University of Sydney, Sydney, Australia, for the careful reading and editing of the manuscript.

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BI9530550