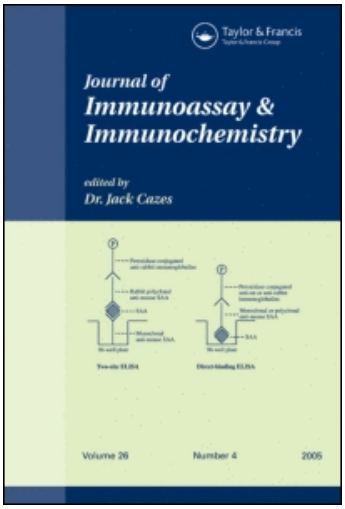

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Characterization of Anti-Advanced Glycation End Product Antibodies to Nonenzymatically Lysine-Derived and Arginine-Derived Glycated Products

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Characterization of Anti-Advanced Glycation End Product Antibodies to Nonenzymatically Lysine-Derived and Arginine-Derived Glycated Products

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Abstract: N^{ε}-(carboxymethyl)lysine (CML) and N^{ε}-(carboxyethyl)lysine (CEL) termed advanced glycation end products (AGEs) are known to be produced by nonenzymatic glycation between bovine serum albumin (BSA) and D-glucose. This study is to characterize the immunoreactivity of anti-AGE antibodies including anti-CML and anti-CEL antibodies. Using AGE-modified BSA (AGE-BSA) as an immunogen, a polyclonal anti-AGE immunoglobulin G (IgG) was produced. The anti-AGE IgG could strongly detect AGEs formed on BSA, at least in part, AGEs produced on both residues Lys and Arg due to its immunoreaction with Lys-derived and Arg-derived AGEs produced by $NaCNBH_3$, a reducing agent, in amino acid glycation analysis, but the pre-immune serum could not. As the anti-CML antibody could also strongly react with AGE-BSA, this suggests that CML is a major nonenzymatically glycated product cross-linked to BSA. Furthermore, CEL is associated with distinguishable polymerization of BSA from CML polymerization of BSA, though weaker than CML and was not produced by Lys glycation analysis. These results indicate that the anti-AGE antibody is

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effective for detecting both Lys-derived and Arg-derived AGEs, and CML and CEL distinctively polymerize albumin as major AGEs present on AGE-BSA.

Keywords: Advanced glycation end products, AGE-modified BSA, N^{ε}-(carboxyethyl)lysine, N^{ε}-(carboxymethyl)lysine, Nonenzymatic glycation, Polymerization

INTRODUCTION

Carbohydrates, especially reducing sugars, react nonenzymatically with proteins and/or lipids to reversibly form Schiff bases, followed by the irreversible formation of Amadori products. These can be broken down into reactive advanced-glycated dicarbonyl compounds termed advanced glycation end products (AGEs).[1,2] These nonenzymatically formed glycated reaction are also called "Brown reaction" or "Maillard reaction" due to their distinct color *in vitro* and their discovery by Maillard, respectively.[1] AGE formation is associated with a number of different disorders including diabetes, atherosclerosis, renal failure, and vascular dysfunctions.[1,3–6] AGEs accumulate in pathological regions associated with different disease states and are also known to deposit in normal tissues during the aging process, indicating that AGEs are closely associated with both disease progress and normal aging.[7] The shapes of AGEs vary according to the kind of amino compounds and sugars involved in the reaction. AGEs are formed on the side chain amino residues of amino acids such as Lys or Arg.[1] N^{ε}-(carboxymethyl)lysine (CML) is the major known AGE produced under diverse reaction conditions, and AGEs such as N^{ε}-(carboxyethyl)lysine (CEL), pentosidine, pyrraline, and imidazolone derivatives have also been identified.[1,8] It was reported that production of CML is closely related to the progress of diverse disease states and has been shown to accumulate in associated pathological tissues via immunochemical approaches.[9] This type of immunochemical study has benefited from the expanded production of different anti-AGE antibodies.[10–14] In the recent studies, anti-CML specific, anti-CEL specific, and glycoaldehyde-modified adduct specific antibodies have been reported.[12–14] It was reported that CEL adduct, methylglyoxal-derived structure of AGEs, can be generated from glucose-modified AGE-BSA even though the relative content of CML is predominant during the course of AGE-BSA formation.[15] These studies demonstrate the usefulness of anti-AGE antibodies, especially in research investigating the pathological mechanisms related to AGE-associated disorders. Thus, this study is to characterize the immunoreactivity of anti-AGE antibodies including CML and CEL antibodies that have less been characterized by the manufacturer.

In this study, AGEs were generated based on reactions involving bovine serum albumin (BSA). An anti-AGE immunoglobulin G (IgG) was produced using this AGE-BSA product as an immunogen, and was characterized by its immunoreactivity to Lys-derived and Arg-derived AGEs. And, it was shown that CML and CEL could distinctively cross-link and polymerize BSA as AGEs that was formed on BSA using different anti-AGE antibodies.

EXPERIMENTAL

Preparation of in Vitro AGE-Modified BSA and Reduced Glycated Products

AGE-modified BSA (AGE-BSA) was prepared as described previously.[10] Briefly, BSA (bovine serum albumin, 1.6 g, Sigma, USA) was dissolved with 3.0 g of D-glucose (Sigma, USA) in 10 mL of 0.5 M sodium phosphate buffer (pH 7.4). The solution was deoxygenated with nitrogen gas and sterilized by ultrafiltration (0.45-μ syringe filter). The sample was incubated at 37°C for 90 days, and thereafter dialyzed against 20 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl. The sample was then lyophilized to yield brown products. As a control, BSA was incubated in parallel without glucose.

For the preparation of amino acid-derived reduced glycated products, each 0.5 M of Lys (K) and Arg (R), and the mixtures of two amino acids at the fixed ratios (R:K = 8:2, or 5:5, or 2:8) were incubated with 0.5 M glyoxylic acid in the presence or absence of 150 mM $NaCNBH_3$, a reducing agent, in 1.5 ml of 0.2 M sodium phosphate buffer (pH 7.4) at 37°C for 2, 4, 6, or 8 days.[8] BSA (250 mg) was dissolved with 45 mM glyoxylic acid in the presence or absence of 150 mM $NaCNBH_3$ in 5.0 ml of 0.2 M sodium phosphate buffer (pH 7.4), then incubated for 1, 2, or 4 days. Each combination of two amino acids and BSA were incubated in the parallel without glyoxylic acid as controls.

Production of Polyclonal Anti-AGE Antibody

A rabbit was immunized with AGE-BSA eight times, similar to previous reports.[10] The immunization was performed using Freund's complete and Freund's incomplete adjuvants following the collection of pre-immune serum. 1.0 mg of the immunogen mixed with 50% Freund's complete adjuvant was injected intradermally at 10 different skin sites. After two weeks, a booster injection was intradermally performed with 1.0 mg of the immunogen in 50% Freund's complete adjuvant. Thereafter, an

additional five booster injections were performed per week. One week after the last booster immunization, 2.0 mg of the immunogen mixed with 50% Freund's incomplete adjuvant was finally injected intradermally and intramuscularly at different skin sites (a total of 15 skin sites). Blood from the rabbit was taken 10 days after the final injection and then antiserum was pooled.

Purification of Anti-AGE IgG

The anti-AGE IgG was purified, with some modifications, as in previous reports.[10,16] IgGs were first purified from the acquired serum by protein A affinity chromatography similar to that described previously.[16] In brief, 10 ml of the serum was loaded onto 10 mL of protein A-Sepharose CL-4B (Sigma, USA). After extensive washing with 20 mM sodium phosphate buffer (pH 7.0), bound IgGs were eluted with 0.1 M citric acid (pH 2.5) and then neutralized with 1.0 M Tris-HCl buffer (pH 10.0). For the purification of the anti-AGE IgG from the IgG pool, the purified IgGs were applied to a BSA-coupled cyanogen bromide-activated Sepharose 4B (Sigma, USA) column and then washed with 20 mM sodium phosphate buffer (pH 7.0). The buffer was pooled and then applied again to the new column equipped with the same BSA-coupled gel, then washed with buffer. The buffer was pooled and then applied to an AGE-BSA-coupled cyanogen bromide-activated Sepharose 4B column. Subsequently, the bound IgG was eluted with 0.1 M citric acid (pH 2.5) and then neutralized with 1.0 M Tris-HCl (pH 10.0). After the purified anti-AGE IgG was concentrated, a titration was measured using ELISA to determine the final concentration.

Western Blot

AGE-BSA and BSA were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After transfer to a nitrocellulose membrane (Pharmacia Biotech, USA), the membrane was blocked with 5% skim milk in 20 mM Tris·HCl (pH 7.5) and 150 mM NaCl containing 0.1% Tween-20 (0.1% TBST) at room temperature for 1 h and then incubated with rabbit polyclonal anti-AGE IgG (10 μg), mouse monoclonal anti-CML antibody (NF-1 G, TransGenic Inc, Japan), or mouse monoclonal anti-CEL antibody (KNH-30, TransGenic Inc, Japan) at 4°C overnight. After washing with 0.1% TBST, the membranes were incubated with anti-rabbit IgG-horseradish peroxidase (HRP) or anti-mouse IgG-HRP antibodies (1:5,000, Pierce, USA), and the antigen-antibody complexes were visualized using SuperSignal West Pico (Pierce, USA).

ELISA Analysis

Each well was coated with 100 μl of AGE-BSA (serial dilutions or 10 μg as indicated) in 50 mM sodium carbonate buffer (pH 9.6) at 4°C overnight. After washing with 0.05% PBST (phosphate-buffered saline containing 0.05% Tween-20), the well was blocked with 250 μl of 0.5% gelatin in 5 mM sodium carbonate buffer (pH 9.6) for 1 h. After washing with 0.05% PBST, the wells were incubated with 10 μg of anti-AGE IgG, 2 μg of anti-CML or anti-CEL antibodies in 100 μl of PBS, or with different doses of anti-AGE IgG in 100 μL of PBS for 1 h. Each well was reacted with biotinylated anti-rabbit (or anti-mouse) IgG and then avidin-biotin HRP complexes. This was followed by the reaction mixture containing 0.05% hydrogen peroxide in 1.0 mL of 0.1 M 1,2-phenylenediamine dihydrochloride and 9.0 mL of 0.05 M Tris-HCl (pH 7.4). The reaction was terminated by addition of 1 M H_2SO_4. The absorbance at 492 nm was read on a micro ELISA plate reader.

The ELISA was also applied toward the quantitation of the anti-AGE IgG, with some modification, as previously described.[17] Briefly, the anti-AGE IgG was serially diluted into each well and assayed as above. The standard curve was obtained with five serial dilutions of the standardized rabbit IgG. After the absorbance was read at 492 nm, the concentration was calculated.

Dot Blot Analysis

After the indicated incubation times, each sample was loaded on nitrocellulose membrane followed by dot blot analysis. After the membrane was blocked with 5% skim milk in 20 mM Tris · HCl (pH 7.5) and 150 mM NaCl at room temperature for 1 h and then followed the procedures as indicated in Western blot.

Statistical Analysis

The data were expressed as the mean ± SEM of three independent experiments. The significance was assessed by student *t*-test. In all the analyses, differences were considered significant at $p < 0.05$ or $p < 0.01$.

RESULTS AND DISCUSSION

Polymerization of BSA by AGEs

Lyophilized AGE-BSA and BSA were run on a SDS-PAGE and then transferred to a nitrocellulose membrane. The blot was incubated with

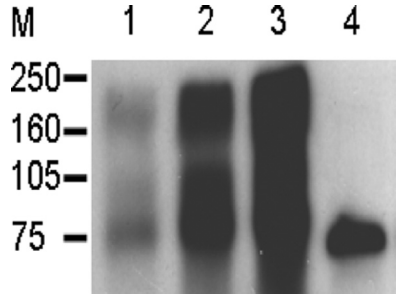

Figure 1. Immunoreactivity of anti-AGE IgG against the immunogen AGE-BSA. BSA (lane 4; 1 μg) incubated alone for 90 days and BSA incubated with D-glucose (AGE-BSA) for the same number of days (lane 1; 1 μg, lane 2; 2 μg, and lane 3; 4 μg) were separated on a 12% polyacrylamide gel and then immunoblotted with anti-AGE IgG. It is observed that anti-AGE IgG immunoreacts in a dose–dependent manner with AGE-cross-linked BSA (AGE-BSA). Note the immunoreaction of the anti-AGE IgG against BSA itself (lane 4). Molecular weights (M, kDa) are shown to the left of the Figure.

the anti-AGE IgG produced in this study (Figure 1). Polymerized and dragged BSA cross-linked by AGEs as well as BSA was detected using the anti-AGE IgG, indicating the possibility that both AGEs formed on BSA and BSA itself may have acted as antigenic material (Figure 1). The rabbit pre-immune serum did not react with AGE-BSA (data not shown). BSA-coupled CN-4B gel and AGE-BSA-coupled CN-4B gel were used to separate the anti-BSA IgG from the anti-AGE IgG. However, the anti-BSA IgG was not completely removed during this process, as shown in Figure 1.

To completely remove BSA immunoreactivity from the anti-AGE IgG, anti-AGE IgG (10 μg) was preincubated with BSA (1.0 g) prior to immunoblot analysis at room temperature for 2 h. The preincubated anti-AGE IgG detected the polymerized BSA cross-linked by AGEs but not BSA itself, indicating the specificity of preincubated anti-AGE IgG towards AGEs (Figure 2).

Immunoreaction of Anti-AGE IgG Against AGE-BSA

Each well was incubated with either a serial dilution or the same concentration (10 μg) of AGE-BSA. The dose-dependent immunoreactivities of anti-AGE IgG against AGE-BSA were observed (Figures 3a and b). In addition, it was observed that the anti-CML antibody strongly reacts in a concentration dependent manner towards AGE-BSA, whereas the anti-CEL antibody shows only faint immunoreactivity (Figure 3c).

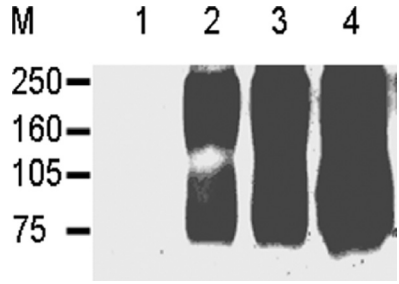

Figure 2. Blocking of anti-BSA IgG activity from anti-AGE IgG by preincubation of anti-AGE IgG with BSA. BSA (lane 1; 1 μg) incubated alone for 90 days and BSA incubated with D-glucose (AGE-BSA) for the same number of days (lane 2; 1 μg, lane 3; 2 μg, and lane 4; 4 μg) were separated on a 12% polyacrylamide gel and then immunoblotted with anti-AGE IgG (10 μg) preincubated with BSA (1.0 g) for 2 h at room temperature. This preincubation effectively blocked the immunoreactivity of anti-AGE IgG against BSA (lane 1) but still demonstrated a dose-dependent immunoreaction of towards AGE-BSA (lane 2–4). Molecular weights (M, kDa) are shown to the left of the figure.

Distinctive Polymerization of Albumin by CML and CEL Through Nonenzymatic Glycation

CML, a type of Lys-derived AGE, is a major AGE formed during the irreversible reaction of nonenzymatic glycation.[8] Anti-CML and anti-CEL antibodies were used to investigate whether Lys residues are involved with the AGE-BSA produced in this study (Figure 4). Strong sustained-dragged CML immunoreactivity was observed against the AGE-BSA polymer, similar to the anti-AGE IgG activity produced in this study, indicating that CML polymerized BSA in the formation of AGE-BSA polymer (Figure 4a). The CEL antibody immunoreacted with broken-dragged AGE-BSA polymer compared to CML polymer (slight bigger AGE-BSA polymer than BSA), though with much weaker immunoreactivity than the CML antibody, and recognized the glycated BSA species with higher molecular weight entrapped into the 4% polyacrylamide stacking gel (Figure 4b). These results indicate that CML cross-links and polymerizes albumin protein being formed on the Lys residues of BSA, and though to a lesser extent, CEL gives a distinguishable contribution from CML to the formation of BSA polymer.

Lys- and Arg-Dependent Glycation on BSA and Implication of Lys Content in Amino Acid Glycation

This study next investigated whether residue Lys and Arg are actually related to AGE formation on BSA. Each glycated amino acid combinations

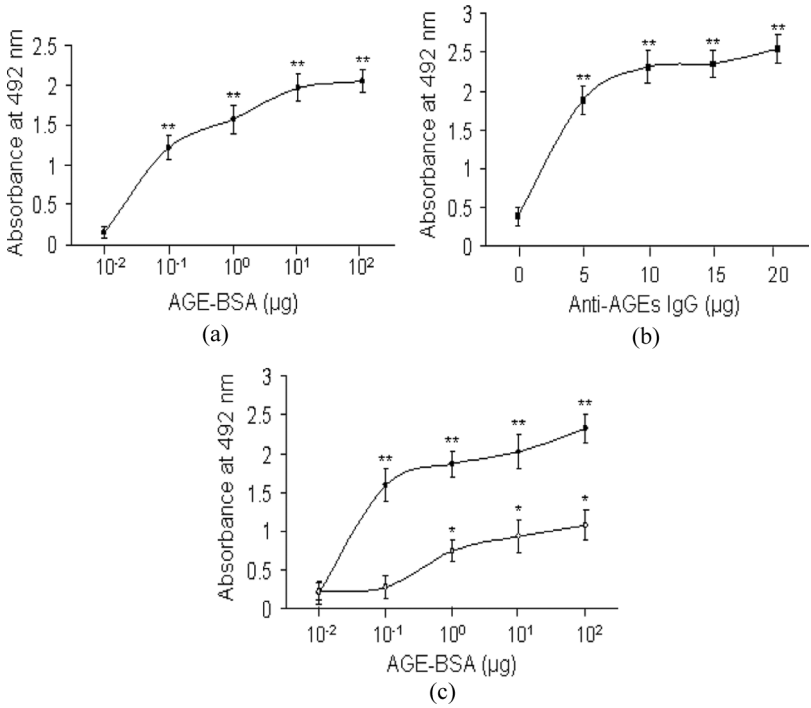

Figure 3. ELISA analysis demonstrating immunoreactivity of anti-AGE, anti-CML, and anti-CEL antibodies towards AGE-cross-linked BSA. As describe in the Materials and Methods, each well was coated with 100 μL of a serial dilution (a and c) or 10 μg (b) of AGE-BSA in 50 mM sodium carbonate buffer (pH 9.6) at 4°C overnight. The wells were incubated with 10 μg of anti-AGE IgG (a), different dilutions of anti-AGE IgG (b), 2 μg of anti-CML (black circle), or 2 μg of anti-CEL (white circle) antibodies (c) in 100 μL of PBS for 1 h. The absorbance at 492 nm was read on a micro ELISA plate reader. The values are expressed as the mean ± SEM of three independent experiments. $^{*}p < 0.05$ or $^{**}p < 0.01$ compared to the control as assessed by student *t*-test.

were first loaded on 3 M paper (Figure 5a). Amino acid combinations incubated with glyoxylic acid showed brown-yellow color according to increased Lys content, and yellow color according to increased Lys content in the presence of a reducing agent, whereas controls (amino acid combinations only) did not show any color reaction (Figure 5a). Lys, Arg and the mixtures of two amino acids at the fixed ratios (Arg (R) : Lys (K) = 8:2, 5:5, or 2:8) were loaded on membrane followed by dot blot analysis using anti-AGE, anti-CML and anti-CEL IgGs (Figures 5b, c, d). Non-glycated amino acid combinations were not recognized by AGE, CML and CEL antibodies (Figures 5b, c, d, line 1, 4, 7 and 10).

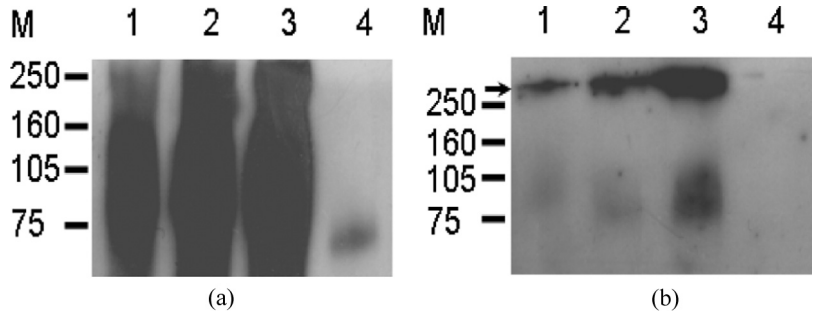

Figure 4. CML and CEL immunoreactivities towards AGE-cross-linked BSA. BSA (lane 4; 1 μg) incubated alone for 90 days and BSA incubated with D-glucose (AGE-BSA) for the same number of days (lane 1; 1 μg, lane 2; 2 μg, and lane 3; 4 μg) were separated on a 12% polyacrylamide gel and then immunoblotted with anti-CML (a) and anti-CEL antibodies (b). The anti-CML antibody shows a strong dose-dependent immunoreactivity against AGE-BSA (a, lane 1–3) and is similar to the immunoreaction against BSA by anti-AGE IgG produced in this study (a, lane 4). In contrast, the anti-CEL antibody does not react against BSA (b, lane 4) and shows a much weaker immunoreactivity than the anti-CML antibody towards AGE-BSA (b, lane 1–3). Molecular weights (M, kDa) are shown to the left of the Figure. After running the gel, the 4% polyacrylamide stacking gel was removed. Arrow indicates the start of the 12% polyacrylamide gel.

Arg-derived, Lys-Arg combinations-derived and Lys-derived AGEs were detected by anti-AGE antibody from 2 days incubation in the presence of a reducing agent, which indicates that anti-AGE antibody produced in this study immunoreacts with both Lys-derived and Arg-derived AGEs (Figure 5b, line 3, 6, 9 and 12). When a reducing agent was not added, there were no immunoreactions for the anti-AGE antibody as similar to background blot density (Figure 5b, line 2, 5, 8 and 11). In contrast, anti-CML antibody immunoreacts with Lys-derived AGE according to increased Lys content in the absence of a reducing agent even weakly, which indicates the inhibition and the retardation of Lys-derived AGE formation in the absence of a reducing agent (Figure 5c, line 2, 5, 8 and 11). CML antibody strongly reacted with Lys-derived AGE according to increased Lys content in the presence of a reducing agent, showing that Lys-derived AGE would certainly be CML (Figure 5c, line 3, 6, 9 and 12). There were no immunoreactions for anti-CEL antibody, which indicates that CEL is not formed in these amino acid combinations-derived AGE products (Figure 5d). In a previous report, CML is well known to be formed from glycated BSA reaction in the presence of a reducing agent.[8] Anti-AGE antibody strongly immunoreacted with BSA-glyoxylic acid glycated products in the presence of a reducing agent according to the incubated times, showing that anti-AGE antibody can detect CML (Figure 5e).

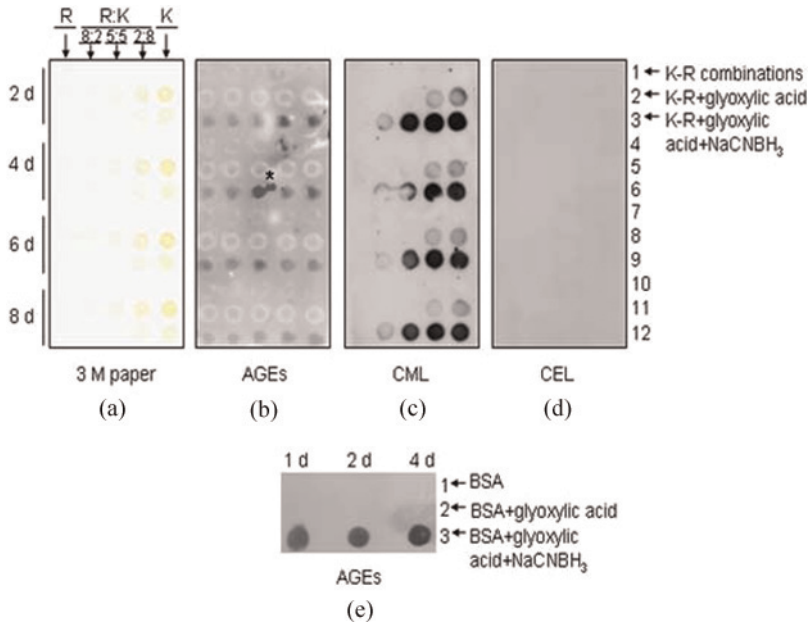

Figure 5. Immunoreaction of anti-AGE antibodies with Lys-derived, Arg-derived and their combinations-derived AGEs. In a–d panel, Arg(R) only, Lys(K) only and the mixtures of two amino acids at the fixed ratios (R:K = 8:2, or 5:5, or 2:8) were used. Amino acid combinations only (line 1, 4, 7 and 10 in a–d panel) and amino acid combinations glycated with glyoxylic acid in the presence (line 3, 6, 9 and 12 in a–d panel) or absence (line 2, 5, 8 and 11 in a–d panel) of $NaCNBH_3$ were incubated for 2, 4, 6 or 8 days. Each sample was dot-loaded on 3 M paper (a) or on membrane (b, c and d), and then the paper was dried and scanned (a). The membrane was used for dot blot analysis using anti-AGE (b), anti-CML (c) and anti-CEL (d) IgGs. BSA only (e, line 1) and BSA glycated with glyoxylic acid in the presence (e, line 3) or absence (e, line 2) of $NaCNBH_3$ were incubated for 1, 2 or 4 days, and then dot blotted with anti-AGE IgG (e). The asterisk in panel b indicates non-specific spot (figure is available in color online).

AGEs are formed by nonenzymatic glycation between amino compounds and sugars, and the different structures associated with AGEs are generated from a wide range of carbohydrates and amino compounds.[18] During the course of glycation, CML adduct can be produced through diverse reaction pathways.[18,19] CEL adducts can be produced from glucose-derived AGE-BSA even though the content of CML is predominant.[15] In previous reports, the contents of CML and CEL in AGE-BSA were traditionally quantified via acid hydrolysis and high performance liquid chromatography (HPLC) equipped with an ion exchange column,

which has also been used previously to investigate the association of AGEs with AGE-related disorders such as diabetes.[13,15,20,21] Enzyme-linked immunosorbent assay (ELISA) has also been previously utilized to evaluate the level of AGEs, following the development of the anti-AGE antibodies.[10,22] These reports showed the formation and the relative contents of AGEs including CML and CEL, but the contribution of CML and CEL to the polymerization of protein is not clear through the successive cross-linking of the protein in the formation of AGE-modified protein.

Recently, both a polyclonal anti-CML antibody and a monoclonal anti-CML antibody (6D12), produced in a preliminary experiment using AGE-BSA, were shown to cross-react with CEL.[12] It appears that these anti-CML antibodies can cross-react with CEL, but it is possible that an anti-CEL specific antibody may coexist within the population of the polyclonal anti-CML antibody. This is likely because a small amount of CEL can be readily formed during the production of AGE-BSA. Supporting this idea, it was shown that the CEL-positive activity in the polyclonal anti-CML antibody sample could be removed by CEL-conjugated affinity chromatography.[12,15] The monoclonal anti-CML antibody (6D12) also cross-reacted with CEL, but recently a CML-specific antibody (CMS-10) was produced.[12]

As with the previous studies, ELISA analysis was applied in this study.[10,22] In addition, Western blot analysis was also used because it helps to distinguish the possibility of AGE-specific cross-reactions more so than ELISA alone. AGE-BSA was recognized by the monoclonal anti-CML and monoclonal anti-CEL antibodies via Western blot analysis and the results indicated a significant difference in the strength and the aspect of their immunoreactivities, as shown in Figure 4. It is possible that the monoclonal anti-CML antibody recognizes CEL on AGE-BSA (as shown in Figure 4a). However, it seems that the monoclonal anti-CEL antibody does not recognize CML (as shown in Figure 4b) because the aspect of the CEL immunoblot should be similar to that of the CML blot if a true cross-reaction is occurring. On the other hand, it should be noted that a CML-positive activity might coexist within the anti-CEL antibody population, even though the anti-CEL antibody is a monoclonal antibody, and that the weak cross-reaction of the anti-CEL antibody to CML cannot be absolutely excluded. The difference in immunoreactivity of the monoclonal anti-CML and anti-CEL antibodies indicates that CML may be a major AGE product associated with AGE-BSA, and that CEL is also formed in a relatively small quantity, and that CML and CEL differently participate in the albumin polymerization by the glycated reaction. The development of anti-AGE antibodies serves to extend immunochemical research associated with AGE-related disorders, and is essential for the evaluation of the relative levels of AGEs, such as CML and CEL.

CONCLUSIONS

In this study, a strong polyclonal anti-AGE antibody that can detect both Lys-derived and Arg-derived AGEs was produced using the immunogen AGE-BSA. It was also shown that the anti-CML antibody (NF-1 G) strongly recognized AGE-BSA and Lys-derived AGEs, definitely CML, whereas the anti-CEL antibody (KNH-30) only weakly detected AGE-BSA, but not Lys-derived AGEs. These results show that CML cross-links and polymerizes albumin as a major structural component of AGEs formed on the AGE-BSA, and CEL is also coupled, though to a lesser extent, to the AGE-BSA. In addition, it is indicated that the aspects of the polymerization of CML and CEL are different, and that the anti-AGE antibody can react with AGEs produced on residues Lys and Arg of BSA, and may contain both anti-CML IgG and anti-CEL IgG populations.

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