Glutaraldehyde is an Effective Cross-linker for Production of Antibodies Against Advanced Glycation End Products

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Abbreviations: AGE(s), advanced glycation end products; HSA, human serum albumin; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; BS3, bis(sulfosuccinimidyl)suberate; CML, N^{ε}-(carboxymethyl)lysine; CEL, N^{ε}-(carboxyethyl)lysine; GO, glyoxal; MG, methylglyoxal; 2SC, *S*-(2-succinyl)cysteine; CMC, *S*-(carboxymethyl)cysteine; CMA, N^{ω}-(carboxymethyl)arginine. ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline

Abstract

Immunohistochemical approaches have been widely used in the localization and quantification of advanced glycation endproducts (AGEs). Traditional approaches for production of anti-AGE antibodies use cross-linkers such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) to conjugate the AGE antigen to the carrier protein. However, these approaches often fail to produce antibodies that are specific to the particular AGE of interest. In the present study, N^{ϵ}-(carboxymethyl)lysine (CML), a major antigenic AGE structure, was conjugated to human serum albumin (HSA) using various cross-linkers; including EDC, bis(sulfosuccinimidyl)suberate (BS3) and glutaraldehyde, to compare their efficiency for the production of epitope specific antibodies. All of the cross-linkers tested were capable of conjugating CML to HSA, and each CML-conjugated HSA was recognized by previously characterized anti-CML antibody. However, only the use of glutaraldehyde as the cross-linker resulted in the production of a CML-specific monoclonal antibody; termed 2G11. 2G11 significantly recognized CML-modified HSA and peptide, whereas it did not recognize N^{ϵ}-(carboxyethyl)lysine (CEL)-modified HSA and peptide, indicating that 2G11 is highly specific to CML, being able to distinguish the difference of a single methyl group between the two epitopes. To further demonstrate the use of glutaraldehyde, anti-AGE antibodies against CEL, *S*-(2-succinyl)cysteine and *S*-(carboxymethyl)cysteine were obtained by conjugation with glutaraldehyde. These studies demonstrate the efficacy of glutaraldehyde as a cross-linker for the production of antibodies against small molecules.

Key words: Monoclonal antibody, Cross-linker, Glutaraldehyde, Advanced glycation end products (AGEs)

1. Introduction

Incubation of proteins with reducing sugars such as glucose leads, through the formation of Schiff bases and Amadori products, to the generation of advanced glycation end products (AGEs). Immunohistochemical studies using the monoclonal anti-AGE antibody (6D12) demonstrated the presence of AGE-modified proteins in several diseased human tissues; including kidneys of patients with diabetic nephropathy (Makino et al., 1995) and chronic renal failure (Yamada et al., 1994), atherosclerotic lesions in the arterial wall (Kume et al., 1995), amyloid fibrils in hemodialysis-related amyloidosis (Miyata et al., 1993; Miyata et al., 1996) and actinic elastosis of the photo-aged skin (Mizutari et al., 1997). Subsequent studies by Koito *et al*. (Koito et al., 2004) demonstrated that 6D12 cross-reacts with both N^{ϵ}-(carboxymethyl)lysine (CML) and N^{ϵ}-(carboxyethyl)lysine (CEL).

Several AGE-specific structures are well characterized, including pyrraline (Hayase et al., 1989), pentosidine (Sell and Monnier, 1989), crosslines (Ienaga et al., 1995), CML (Ahmed et al., 1986), CEL (Ahmed et al., 1997), GA-pyridine (Jono et al., 2002), OP-lysine (Argirov et al., 2004), glyoxal-lysine dimer (GOLD) and methylglyoxal-lysine dimer (MOLD) (Frye et al., 1998), imidazolone (Niwa et al., 1997), and vesperlysines A, B and C (Nakamura et al., 1997). After the anti-AGE antibody was reported in 1989 (Nakayama et al., 1989), the detection of AGEs using the antibodies has spread widely. Especially, it has been believed to be a useful tool to detect the acid labile AGE structures such as imidazolone (Jono et al., 2004), which are destroyed during conventional acid hydrolysis with 6 N HCl at 110°C for 24 h. However, Thornalley et al. (Thornalley et al., 2003) successfully detected and quantified those AGE structures by liquid chromatography with tandem mass spectrometric detection after enzymatic digestion. Although the quantification of AGEs by instrumental analyses is superior to immunological analyses,

anti-AGE antibodies can be a convenient means for estimating AGE contents and examining the histological localization of AGEs. Previous work by our group, demonstrated that CML is localized within intracellular lesions of foam cells and extracellular matrix in human atherosclerotic lesions (Kume et al., 1995). Whereas GA-pyridine mainly localized in intracellular lesion of foam cells (Jono et al., 2002). These studies indicating that histological localization of each AGE is different even in the same organ. Therefore, the use of an epitope-specific anti-AGE antibody is required to elucidate the differential distribution and pathophysiological significance of AGEs.

Standard cross-linking approaches for the conjugation of AGE to carrier protein have been used in the production of anti-AGE antibodies.
1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) produces a peptide bond between hapten and carrier protein, and is widely used to develop antibodies against haptens (Grabarek and Gergely, 1990). Using this technique, we developed antibodies against imidazolone (Jono et al., 2004), pentosidine (Miyazaki et al., 2002) and nitrotyrosine (Nagai et al., 2002). However, past attempts at producing antibodies against CML and CEL using EDC have failed. In contrast, glutaraldehyde forms Schiff base crosslinks between amino residues of the hapten and carrier protein; these are then reduced to a stable secondary amine. Glutaraldehyde is widely used for the production of antibodies against macromolecules such as peptides (Gullick, 1994), but has not been commonly used for the production of antibodies against small molecules.

In the present study, we compared the effectiveness of 3 cross-linkers in the production of antibodies against various AGEs. Our results demonstrate that glutaraldehyde is a promising cross-linker for production of antibodies against AGEs, providing an inexpensive procedure which yields a robust AGE-specific response.

2. Materials and methods

2.1. Materials

Human serum albumin (HSA) was donated by Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan) and was defatted using charcoal treatment as described by Chen (Chen, 1967). EDC, *N*-hydroxysulfosuccinimide and bis(sulfosuccinimidyl)suberate (BS3) were purchased from Pierce (Rockford, IL). Glutaraldehyde was purchased from Kanto Chemical (Tokyo, Japan). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody was purchased from Kirkegaard Perry Laboratories (Gaithersburg, MD). Mouse monoclonal antibody isotyping test kit was purchased from Serotec (Oxford, UK). *S*-(carboxymethyl)cysteine (CMC) was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were of the best grade available from commercial sources.

2.2. Preparation of CML-modified HSA and CEL-modified HSA

CML-modified HSA with increasing levels of CML was prepared as described previously (Ikeda et al., 1996). Briefly, HSA (50 mg/ml) was incubated at 37°C for 24 h with 0.4-100 mM glyoxylic acid and 100 mM sodium cyanoborohydride ($NaBH_3CN$) in 1 ml of 0.2 M sodium phosphate buffer (pH 7.8), followed by dialysis against phosphate buffered saline (PBS). CEL-modified HSA was prepared similarly by incubating HSA (50 mg/ml) with 0.4-100 mM pyruvate at room temperature for 12 h in the presence of 5 mM $NaBH_3CN$ in 1 ml of 0.2 M sodium phosphate buffer (pH 7.4), followed by dialysis against PBS (Ahmed et al., 1997). CML-modified keyhole limpet hemocyanin (KLH) and CEL-modified KLH were prepared as described above. The CML and CEL content of the samples was determined by amino acid analysis after acid hydrolysis (6 N HCl for 24 h at 110°C) using an amino acid analyzer (L-8500A, Hitachi) (Tokyo, Japan) equipped with an

ion exchange column (#2622 SC, 4.6 x 80 mm, Hitachi), as described previously (Matsumoto et al., 2000). Hippuryl-CML (Ikeda et al., 1996) and hippuryl-CEL (Nagai et al., 2003) were prepared as described previously.

2.3. Conjugation of AGE-molecules to proteins with cross-linkers

CML was prepared by overnight incubation of 0.26 M Nα-acetyllysine 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) at room temperature as described previously (Knecht et al., 1991).

EDC method (Miyazaki et al., 2002): 2 mg of CML was incubated with 8 mg of HSA in 4 mL of PBS in the presence of 50 mM EDC and 2.5 mM N-hydroxysulfosuccinimide at room temperature for 1 h. The reaction was terminated by the addition of 1/1000 (v/v) of 2-mercaptoethanol and the sample was dialyzed against PBS for 24 h at 4°C.

BS3 method (Mattson et al., 1993): 5 mg of CML was incubated with 5 mg of HSA in 1 mL of PBS in the presence of 5 mM BS3 at room temperature for 1 h. The reaction was terminated by the addition of 1/10 (v/v) of 1M glycine and the sample was dialyzed against PBS for 24 h at 4°C.

Glutaraldehyde method: 5 mg of CML was incubated with 5 mg of HSA in 1 mL of PBS in the presence of 50 mM glutaraldehyde at room temperature for 1 h. The reaction was terminated by the addition of 1/10 (v/v) of 1M glycine and the sample was dialyzed against PBS for 24 h at 4°C. CML-conjugate KLH and lysine-conjugate HSA were prepared by the same method.

2-succinylcysteamine (2SCEA) was synthesized according to Nagai *et al*. (Nagai et al., 2007). Briefly, cysteamine (500 mM) was incubated with a 10% molar excess of

N-ethylmaleimide (NEM) in phosphate buffer (200 mM, pH 7.4) for 1 hr at room temperature. 2SCEA was recovered by hydrolysis in 6 M HCl for 6 hr at 110 °C, followed by desalting on a Dowex-50 cation exchange resin, and fractionation on a Pickering (Pickering Labs, Mountain View, CA) sulfonated divinylbenzene cation-exchange column (250 mm x 3 mm) using a Pickering buffer system at a flow rate of 0.35 ml/min. The buffers used were as follows: buffer A (pH 3.28), 97.3% water, 2% sodium citrate, 0.6% HCl; buffer B (pH 7.4), 93.8% water, 5.3% NaCl, 0.8% sodium acetate; buffer C (pH 12.7), 99% water, 0.6% NaOH, 0.4% NaCl. The gradient was as follows: 0-15 min, 100% A; 15-39 min, linear ramp to 100% B; 39-60 min, hold at 100% B; 60-60.1 min, linear ramp to 100% C; 60.1-62.5 min, hold at 100% C; 62.5-62.6 min, return to 100% A; 62.6-83 min hold at 100% A. 2SCEA and CMC were conjugated with glutaraldehyde to HSA and KLH as described above for CML.

2.4. Preparation of monoclonal antibody specific for CML

The experimental protocol was approved by the local ethics review committee for animal experimentation. First, 100 μg of each CML-conjugated HSA in 50% Freund's complete adjuvant was injected intradermally in Balb/c mice, followed by two booster injections with 100 μg of CML-conjugated HSA in 50% Freund's incomplete adjuvant. Antibody titers against CML in mice immunized with CML-conjugated HSA were determined by enzyme-linked immunosorbent assay (ELISA). Splenic lymphocytes isolated from the mouse immunized with glutaraldehyde cross-linkde CML-conjugated HSA were fused to myeloma P3U1 cells in the presence of polyethylene glycol, as described previously (Jono et al., 2002). Hybridoma cells positive to CML-modified KLH but negative to CEL-modified KLH and HSA were selected through successive subcloning. One cell line, named 2G11, was produced from the ascitic fluid of Balb/c mice and further

purified on protein G-immobilized Sepharose gel to IgG.

2.5. ELISA

ELISA was performed as described previously (Mera et al., 2007). Briefly, each well of a 96-well microtiter plate was coated with 100 µl of the indicated concentration of sample in PBS, and incubated for 2 hr. The wells were washed three times with PBS containing 0.05% Tween 20 (washing buffer), then blocked with 0.5% gelatin in PBS for 1 hr. After washing 3 times, the wells were incubated for 1 hr with 100 µl of the indicated concentration of primary antibody. After triplicate washing, the wells were incubated with HRP-conjugated anti-mouse IgG, followed by reaction with 1,2-phenylenediamine dihydrochloride. The reaction was terminated with 100 µl of 1.0 M sulfuric acid, and monitored using absorbance at 492 nm with a micro-ELISA plate reader (TECAN Spectra Fluor Plus).

2.6. Human lens samples

The human cataract lens samples were obtained after receiving the approval of the institutional review board of Shinshu University and Kumamoto University, with informed consent from all patients. The fragments of lenses, with cataract (8 from diabetes and 6 from non-diabetes), were collected during surgery. The protein concentration of the samples were measured using a bicinchoninic acid protein assay reagent (Pierce, Rockford, IL). The CML content of each sample was measured by amino acid analysis as described above.

3. Results

3.1. Conjugation of CML to proteins with cross-linkers

CML was conjugated to carrier proteins with cross-linkers such as EDC, BS3 and glutaraldehyde (Fig. 1A) and their conjugating efficacy was determined by noncompetitive ELISA. As shown in Fig. 2, a previously characterized anti-CML antibody (6D12) (Ikeda et al., 1996) significantly reacted with all CML-conjugated HSA and CML-conjugated KLH. A control conjugation with lysine showed negligible reactivity toward, indicating that CML was conjugated to the carrier proteins.

3.2. Antigenic activity of CML-conjugated HSA in mice

The efficacy of EDC, BS3 and glutaraldehyde in the production of antibodies against AGEs was tested by immunization of mice with CML-conjugated HSA. The antigenic activity of all three CML-conjugation preparations was then tested by ELISA. As shown in Fig. 3, all antisera obtained from the immunized mice reacted with HSA (control for immune response). However, only antiserum from the mice immunized with glutaraldehyde-conjugated CML-HSA recognized both CML-modified KLH and CML-conjugated KLH (Fig. 3C), indicating that glutaraldehyde was a significantly more effective cross-linker, compared to EDC and BS3, for the production of antibodies against small molecules.

3.3. Immunoreactivity of the monoclonal anti-CML antibody

Splenic lymphocytes collected from a mouse immunized with glutaraldehyde cross-linked CML-conjugated HSA, were fused to myeloma P3U1 cells, followed by successive screenings. The cell line 2G11 (IgG1 subclass), was positive to CML-modified

KLH, but negative to CEL-modified KLH and HSA. As shown in Fig. 4A, 2G11 significantly reacted with CML-modified HSA in a dose-dependent manner, whereas its reactivity with CEL-modified HSA was negligible. Competitive ELISA also showed that the reaction of 2G11 to CML-modified HSA was significantly inhibited by hippuryl-CML and CML free adduct but not by hippuryl-CEL and CEL free adduct (Fig. 4B). Also, the reactivity of 2G11 to CML-modified HSA significantly correlated with degree of CML modification ($R = 0.993$, $P < 0.001$) (Fig. 4C). This data indicates that the anti-CML antibody 2G11 is a CML specific antibody, as compared to 6D12 which recognizes both CML and CEL (Koito et al., 2004).

Recent studies have shown that the aldehydes, glyoxal (GO) (Koito et al., 2004) and methylglyoxal (MG) (Nagai et al., 2003), react with lysine to form CML and CEL, respectively. Based on this knowledge, we tested the ability of 2G11 to distinguish between GO and MG modified HSA by non-competitive ELISA. As shown in Fig. 4D, 2G11 significantly reacted with GO-modified HSA, while its reactivity with MG-modified HSA was negligible, indicating that 2G11 is capable of distinguishing between CML and CEL, which differ by a single methylene group.

3.4. Immunoreactivity of the 2G11 to human lens samples

There are many compounds in physiological samples that may have cross-reactivity. Therefore, we measured the CML levels in human lens samples by 2G11, and compared those CML levels to that obtained by HPLC. As shown in Fig. 5, the reactivity of 2G11 with human lens proteins was highly correlated with their CML contents, which were determined by HPLC ($R = 0.811$, $P < 0.01$) (Fig. 5). This suggests that 2G11 specifically recognizes CML.

3.4. Preparation of anti-AGE antibodies by coupling with glutaraldehyde

As described in the introduction, several AGE structures have been characterized. Due to the success of 2G11 in the distinguishing of AGEs, we decided to utilize the glutaraldehyde technique in the production of other anti-AGE antibodies. Using this method, we were able to produce specific monoclonal antibodies against CEL, *S*-(2-succinyl)cysteine (2SC), and *S*-(carboxymethyl)cysteine (CMC) (Fig. 1B). A monoclonal anti-CEL antibody (clone 2E1, IgG2b subclass) significantly and specifically reacted with CEL-conjugate HSA, CEL-modified HSA and MG-HSA, but had negligible cross-reactivity with CML, CMC, N^{ω}-(carboxymethyl)arginine (CMA) and GO-HSA (Fig. 6A). Also, the reactivity of 2E1 to CEL-modified HSA significantly correlated with degree of CEL modification ($R = 0.975$, $P < 0.001$) (data not shown). The monoclonal anti-2SC antibody (clone 3B10, IgG1 subclass) was also significantly and specifically reactive toward 2SCE-conjugated proteins and 2SC-modified proteins, but exhibited negligible cross-reactivity with CML, CMC, and native proteins (Fig. 6B). Also, the monoclonal anti-CMC antibody (clone 3F4, IgG1 subclass) significantly reacted with CMC-conjugated HSA, while cross-reactivity with cysteine, CML, CMC, CMA and 2SC was negligible (Fig. 6C). These results illustrate the merits of using glutaraldehyde as a cross-linker for the production of antibody against a variety of small-molecule structures such as AGEs.

4. Discussion

Immunochemical approaches have been widely used in the histological localization and quantification of tissue AGEs. Since the AGEs themselves are modified amino acids with molecular weights of less than 500 Da, it is necessary to conjugate these molecules to carrier proteins, in order to produce antibodies. Although EDC is the most conventional coupling reagent for small molecules and produces peptide bond between carrier protein and hapten, EDC-conjugated hapten-carrier adducts often fail to produce immune responses against small molecule haptens. In the present study, CML, a major antigenic AGE structure, was conjugated to HSA with three different cross-linkers, EDC, BS3 and glutaraldehyde, and their efficacy in the production of antibodies was compared. Although all three CML-conjugated HSAs were strongly recognized by anti-CML antibody (Fig. 2), only CML-conjugated HSA prepared by glutaraldehyde cross-linking produced an antibody against CML (Fig. 3). Antibodies against CEL, 2SC and CMC were also obtained by conjugation to carrier proteins using glutaraldehyde (Fig. 6), indicating that glutaraldehyde is a promising cross-linker for production of antibody against small molecules.

Although the EDC method is commonly used for preparation of antibodies against small molecules, CML-conjugate HSA prepared with EDC failed to produce a viable antibody against CML (Fig.3), CEL or 2SC (data not shown). In addition, BS3 contains an 8-carbon spacer arm, which may induce antigenicity to the linker region instead of the hapten. In fact, the antiserum from mice immunized with CML-conjugated HSA prepared by BS3 reacted not only with CML-conjugated KLH but also with Lys-conjugated KLH (Fig. 3B). These results also emphasize that glutaraldehyde is a significantly more effective cross-linker, compared to EDC and BS3, for the production of antibodies against small molecules such as AGEs.

We previously demonstrated that proteins modified with aldehydes such as glycolaldehyde and glucose show ligand activity for scavenger receptors expressed on the macrophages (Matsumoto et al., 2000; Ohgami et al., 2001a; Ohgami et al., 2001b; Jono et al., 2002) and liver endothelial cells (Matsumoto et al., 2000). It is known that antigenicity of carrier protein is involved in the production of the antibodies against hapten. Taken together, it is speculated that glutaraldehyde-treated HSA may be recognized by macrophages through the scavenger receptors, which result in the high antigenic activity. In fact, although all three cross-linkers were cable of conjugating CML to HSA, only CML-conjugated HSA with glutaraldehyde resulted in the production of monoclonal CML-specific antibody.

The anti-CML antibody 2G11, significantly recognized CML, but not CEL, indicating that the antibody distinguishes the difference of one methylene group (Fig. 4). The reactivity of 2G11 to the CML free adduct was lower than that of hippuryl-CML; it is reported that the reactivity of CML-protein adducts to anti-CML antibody is higher than that of CML free adduct (Reddy et al., 1995). Furthermore, the reactivity of 2G11 to CML modified HSA significantly correlated with the degree of CML modification (Fig. 4), indicating that 2G11 is capable of quantifying the degree of modification on target proteins. We further applied the glutaraldehyde method to produce hapten-specific monoclonal antibodies against CEL, 2SC and CMC (Fig. 6). 2SC has been identified as a chemical modification of cysteine sulfhydryl groups by fumarate, via a Michael addition reaction (Alderson et al., 2006). Recent studies using gas chromatography/mass spectrometry (GC/MS) demonstrate that 2SC increases in human skin collagen with age, and is increased in muscle protein of diabetic vs. healthy control rats. On the other hand, CMC, a sulfhydryl-AGE, was discovered in muscle protein and the levels of CMC were comparable to those of CML and increased similarly in response to diabetes (Alt et al.,

2004). To date, there are no reports showing the specific monoclonal antibodies against 2SC and CMC, however the antibodies produced in this study could be powerful tools in the histological localization and elucidation of the pathophysiological significance of 2SC and CMC.

In summary, this study demonstrates the usefulness of glutaraldehyde as a cross-linking agent for in production of antibodies against small molecules, such as AGEs. Using the method described here, we have developed not only more specific antibodies than conventional antibodies, such as 6D12, but also the antibodies against AGE which had not been previously developed. Therefore, this method will be applied for the production of antibody libraries for AGEs. Future studies will be required to improve the sensitivity of the antibodies, and apply them to the detection of AGEs in physiological samples such as albumin.

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Figure legends

Fig. 1. (A) The Structures of EDC, BS3 and glutaraldehyde cross-linkers used in the conjugation of AGEs to carrier proteins. (B) The structures of AGE used as an antigen in this study.

Fig. 2. Reactivity of CML-conjugated proteins, via EDC, BS3 and glutaraldehyde cross-linking, with a known anti-CML antibody (6D12). CML was conjugated to proteins using the cross-linkers EDC, BS3 and glutaraldehyde, as described in Materials and Methods. Efficacy of conjugation was tested by noncompetitive ELISA, as follows; CML-conjugated proteins (10 μg/mL) were coated on the ELISA plate and incubated for 2 hr. The wells were washed and blocked with gelatin, followed by reaction with 6D12 (0.5 μg/mL). The bound antibody was detected by HRP-conjugated anti-mouse IgG antibody, as described in Materials and Methods. Data are the mean ± SD (n = 3).

Fig. 3. Titer of sera from mice immunized with various forms of CML-conjugated HSA. Antiserum was obtained from CML-conjugated HSA immunized mice, from immunogen prepared using EDC (A), BS3 (B), or glutaraldehyde (C). Samples (10 μg/mL) were coated on the ELISA plate and incubated for 2 hr. The wells were washed and blocked with gelatin, followed by reaction with mouse antiserum. The bound antibodies were detected by HRP-conjugated anti-mouse IgG antibody, as described in Materials and Methods. HSA (open circles), CML-modified KLH using glyoxylic acid (closed squares), CML-conjugated KLH (closed triangles) and Lys-conjugated KLH (open triangles). Data are the mean ± SD (n = 4).

Fig. 4. Immunoreactivity of the 2G11 monoclonal antibody to CML. (A) Each well of a 96-well microtiter plate was coated with various concentrations of CML-modified HSA (open circles) (8.7 mol CML/mol HSA) or CEL-modified HSA (closed triangles) (12.8 mol CEL/mol HSA). Coated wells were incubated with 2G11 (1 μg/mL). The bound antibodies were then detected using HRP-conjugated anti-mouse IgG, as described in the Materials and Methods section. (B) Each well was coated with 0.1 mL of 1 μg/mL CML-modified HSA (3.1 mol CML/mol HSA) and blocked with 0.5% gelatin. The test sample (50 μL) was added to each well in the presence of 50 μL of 2G11 (2 μg/mL), followed by incubation for 60 min. The bound antibodies were detected as previously described. Hippuryl-CML (open circles), hippuryl-CEL (closed circles), CML free adduct (open squares) and CEL free adduct (open squares). (C) The reactivity of 2G11 to CML-modified HSA, prepared with different concentrations of glyoxylic acid, was determined by non-competitive ELISA as previously described. Each well was coated with 100 μl of 10 μg/mL each CML-modified HSA, and 1 μg/mL of 2G11 was used as the primary antibody. The immunoreactivity was plotted against CML contents, determined by amino acid analysis. (D) Each tube, containing 2 mg/mL HSA and 33 mM glyoxal (GO) or 33 mM methylglyoxal (MG) in PBS, was incubated at 37 °C for up to 7 days, and aliquots of each sample were collected at 1, 2, 4 and 7 day, followed by dialysis against PBS. The immunoreactivity of each sample to 2G11 was determined at a fixed concentration of antigen (10 μg/mL).

Fig. 5. Immunoreactivity of the 2G11 to human lens samples. Each well of a 96-well microtiter plate was coated with 0.1 mL of 10 μg/mL human lens samples. Coated wells were incubated with 2G11 (1 μg/mL). The bound antibodies were then detected using HRP-conjugated anti-mouse IgG, as described in the Materials and Methods section. The

immunoreactivity was plotted against the CML contents of each sample, which was analyzed by amino acid analysis.

Fig. 6. Immunoreactivity of the monoclonal antibodies against CEL, 2SC, and CMC. The immunoreactivity of the monoclonal antibodies against CEL (2E1) (A), 2SC (3B10) (B), and CMC (3F4) (C) were determined by non-competitive ELISA as described previously. Each well was coated with 100 µl of 10 µg/mL samples, and 2E1 (5 µg/mL), 3B10 (1 µg/mL), or 3F4 (1µg/mL) were used as primary antibodies. CML-, CEL-, 2SCE-, CMC-, and CMA-conjugate proteins were all prepared by the glutaraldehyde cross-linking method. CEL-modified HSA (5.7 mol CEL/mol HSA) and CML-modified HSA (8.7 mol CML/mol HSA) were prepared as previously described in *Materials and Methods*. MG-HSA and GO-HSA were prepared by incubating 2 mg/mL HSA with 33mM MG or GO, respectively, in PBS at 37 °C for 3 days. 2SC-modified proteins were prepared as previously described (Nagai et al., 2007).