

# Autoimmune response to advanced glycosylation end-products of human LDL

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**Abstract** Advanced glycosylation end-products (AGEs) are believed to play a significant role in the development of vascular complications in diabetic patients. One such product, AGE-LDL, has been shown to be immunogenic. In this report, we describe the isolation and characterization of human AGE-LDL antibodies from the sera of seven patients with Type 1 diabetes by affinity chromatography using an immobilized AGE-LDL preparation that contained primarily the AGE N<sup>ε</sup>(carboxymethyl)lysine (CML, 14.6 mmol/mol lysine), and smaller amounts of N<sup>ε</sup>(carboxyethyl)lysine (CEL, 2.7 mmol/mol lysine). The isolated antibodies were predominantly IgG of subclasses 1 and 3, and considered proinflammatory because of their ability to promote FcγR-mediated phagocytosis and to activate complement. We determined dissociation constants ( $K_d$ ) for the purified antibodies. The average  $K_d$  values ( $4.76 \pm 2.52 \times 10^{-9}$  mol/l) indicated that AGE-LDL antibodies are of higher avidity than oxidized LDL antibodies measured previously ( $K_d = 1.53 \pm 07 \times 10^{-8}$  ml/l), but of lower avidity than rabbit polyclonal LDL antibodies ( $K_d = 9.34 \times 10^{-11}$ ). Analysis of the apolipoprotein B-rich lipoproteins isolated with polyethylene glycol-precipitated antigen-antibody complexes from the same patients showed the presence of both CML and CEL, thus confirming that these two modifications are recognized by human autoantibodies. **■** A comparative study of the reactivity of purified AGE-LDL antibodies with CML-LDL and CML-serum albumin showed no cross-reactivity.—Virella, G., S. R. Thorpe, N. L. Alderson, E. M. Stephan, D. Atchley, F. Wagner, M. F. Lopes-Virella, and the DCCT/EDIC Research Group. Autoimmune response to advanced glycosylation end-products of human LDL. *J. Lipid Res.* 2003. 44: 487–493.

**Supplementary key words** modified lipoproteins • diabetes • immunogenicity of advanced glycosylation end-products-LDL • advanced glycosylation end-products-LDL antibodies

It is well known that diabetes mellitus is associated with an increased incidence of macrovascular complications, including coronary heart disease, cerebrovascular disease,

and peripheral vascular disease (1–3). The mechanisms by which diabetes accelerates atherosclerosis are not well understood. It has been proposed that an increased level of chemically modified lipoproteins might be a significant factor contributing to the accelerated development of macrovascular complications in diabetes (2). The persistence of high plasma glucose levels in diabetic individuals creates favorable conditions for some of these modifications to occur, including glycosylation, glycooxidation (2), and advanced glycosylation (3).

Advanced glycosylation involves a chain of chemical reactions that starts with the covalent, nonenzymatic addition of reducing sugars to protein amino groups (Schiff base, Amadori adducts). If the half-life of a protein is sufficiently long, additional reactions take place, leading to the formation of a heterogeneous family of sugar-amino acid adducts collectively known as advanced glycosylation end-products (AGEs) (3). LDL, like all plasma proteins, is susceptible to AGE modification (4). A variety of potentially pathogenic consequences can be linked to AGE modification of LDL, including direct or indirect induction of proinflammatory circuits (4–6), as well as trapping of protein in atherosclerotic plaques (5). In addition, AGE-modified proteins are immunogenic (7), a property that has been used to great advantage for their detection in serum (2) and localization in tissues (2, 8).

The immunogenicity of AGE-modified proteins is not limited to the induction of heterologous antibodies in experimental animals. Autoantibodies to AGE-modified serum albumin and AGE-modified IgG have been demon-

Abbreviations: AGE, advanced glycosylation end-product; ALE, advanced lipoxidation end-product; CEL, (carboxyethyl)lysine; CML, (carboxymethyl)lysine; EDIC, Epidemiology of Diabetes Complication; EIA, enzyme immunoassay; IC, immune complexes; oxLDL, oxidized LDL.

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strated in human sera, both from diabetic patients as well as in nondiabetic subjects (9–11). Data suggesting that these antibodies are able to combine with circulating AGE-modified antigens and form soluble immune complexes (ICs) have also been recently reported (10).

Of particular importance would be the demonstration that AGE-LDL is immunogenic, given the considerable wealth of evidence suggesting that LDL-ICs may play a significant pathogenic role in atherosclerosis (12, 13). LDL-ICs containing oxidized LDL (oxLDL) have been demonstrated in atherosclerotic lesions (14, 15), and studies with model ICs prepared with human LDL and rabbit antibodies have shown that LDL-ICs cause foam cell formation and activate the release of proinflammatory cytokines and matrix metalloproteinases (16–18). Similar properties have also been demonstrated for polyethylene glycol (PEG)-precipitated ICs containing LDL obtained from the sera of diabetic patients (19, 20).

Our group has characterized in detail antibodies reacting with oxLDL isolated from the sera of patients with diabetes and nondiabetic subjects. Those antibodies are predominantly of the IgG isotype restricted to the proinflammatory subtypes 1 and 3 (21, 22). OxLDL antibodies have a moderate  $K_d$  ( $1.1 \pm 1.05 \times 10^{-8}$  mol/l), and cross-reactivity studies showed that they react primarily with malondialdehyde (MDA)-lysine epitopes (23). In this report, we describe the isolation and characterization of AGE-LDL antibodies isolated from seven Type 1 diabetic subjects and the results of experiments aimed at defining the nature of the immunogenic modifications that elicit autoantibody formation. This, to our knowledge, is the first report of the isolation and characterization of human autoantibodies to AGE-LDL.

## MATERIALS AND METHODS

### Sera

The sera from 13 patients with Type 1 diabetes were used as the source of purified AGE-LDL antibodies and modified lipoproteins coprecipitated with antigen-antibody complexes. The serum samples were obtained as part of the Diabetes Control and Complications Trial/Epidemiology of Diabetes Complication (DCCT/EDIC) study at participating EDIC centers. The EDIC study is a continuation of the DCCT study involving patients from the original DCCT cohort who joined the EDIC study. The primary goal of EDIC is to study the development of macrovascular disease in Type 1 diabetes. A collaborative project (Markers and Mechanisms for Macrovascular Disease in insulin-dependent diabetes mellitus) between the Medical University of South Carolina and the DCCT/EDIC Group has as its primary goal the identification of new markers, risk factors, and mechanisms for macrovascular disease in Type 1 diabetes. A secondary goal is to relate putative vascular risk factors to microvascular complications (retinopathy and nephropathy) since it was hypothesized that common mechanisms of vascular damage may be implicated. Informed consent as approved by the Institutional Review Board for Human Research of every Center involved in the DCCT/EDIC trial was obtained from each subject included in this study.

### Isolation of LDL and AGE-modification of isolated LDL

Blood was collected in 0.4 mM EDTA after a 12 h fast. LDL was isolated from individual or pooled plasma after density adjust-

ment ( $1.019 < d < 1.063$  g/ml) with potassium bromide (KBr) by preparative ultracentrifugation at 50,000 rpm for 17 h on a Beckman L-80 ultracentrifuge using a type 70 Ti rotor (19). The isolated LDL was washed by ultracentrifugation, dialyzed against a 0.15 mol/l sodium chloride solution containing 0.3 mM EDTA, pH 8.0, then was passed through an Acrodisc 0.2  $\mu$ m filter in order to sterilize and remove aggregates, and stored under nitrogen in the dark at 4°C.

AGE-LDL was prepared by a modification of the method described by Schmidt et al. (24). Freshly isolated LDL at a 1.2 mg/ml concentration was sterilized by passage through a 0.2  $\mu$ m filter added to 150 mM glucose-6-phosphate in 200 mM phosphate buffer, pH 7.8, containing 40  $\mu$ M butylhydroxytoluene (BHT) and 540  $\mu$ M EDTA, filter-sterilized a second time, and incubated for 8 weeks at 37°C. At the end of the incubation, the LDL solution was dialyzed for 8 h against two changes of 4 liters of 0.15 M NaCl, 0.3 mM EDTA, pH 8.0. The dialyzed AGE-LDL was filter-sterilized a second time, the final protein concentration determined by the Lowry assay (25), and stored in the dark at 4°C.

### Analysis of LDL modification

Analysis of purified lipoproteins for their content of (carboxymethyl)lysine (CML), (carboxyethyl)lysine (CEL), and the advanced lipoxidation end-products (ALEs) MDA- and 4-hydroxynonenal (HNE)-lysine was carried out by isotope dilution selected ion-monitoring gas chromatography-mass spectrometry (SIM-GC-MS) as described by Requena et al. (3). Briefly, these four AGE/ALEs were measured simultaneously in LDL (0.4 mg protein) after borohydride reduction of the protein. The reduced sample was dialyzed, dried, delipidated [chloroform-methanol (2:1, v/v), containing 0.02% BHT], and following addition of heavy labeled internal standards, hydrolyzed at 110°C in 6 N HCl for 18 h. The hydrolysate was dried, passed over a 1 ml solid phase extraction  $C_{18}$ -column (Sep-pak, Waters, Milford MA) to remove brown products formed during hydrolysis, dried again, and then amino acids derivatized as their trifluoroacetyl methyl esters for analysis by SIM-GC-MS (1–3). All modified amino acids are expressed as a ratio to the parent amino acid, lysine.

### Preparation of Sepharose-AGE LDL and isolation of AGE-antibodies

AGE-LDL was coupled to Sepharose using a modification of the method previously described by us for preparation of oxLDL-Sepharose (19, 23). AGE-LDL was dialyzed against coupling buffer (0.1 M  $\text{NaHCO}_3$  + 0.5 M NaCl, pH 8.3) overnight at 4°C, mixed and incubated with activated Sepharose (2 g), and prepared according to the manufacturer's instructions (Amersham-Pharmacia Biotech, Piscataway, NJ) at a protein-dry gel ratio of 12.5 mg/g. Coupling was allowed to proceed at 4°C overnight on an end-over-end mixer. Free reactive sites remaining on the Sepharose gel were blocked with 0.1 M Tris buffer, pH 8.0, and, after extensive washing and degassing, the gel suspension (7 ml) was transferred to a chromatography column and washed thoroughly with 0.01 M  $\text{NaHCO}_3$  pH 8.3 buffer.

AGE-LDL antibodies were isolated from 1 ml aliquots of patient sera diluted in 3 ml of 0.01 M  $\text{NaHCO}_3$  pH 8.3 buffer. The diluted serum was loaded at room temperature into the AGE-LDL-Sepharose column. The column was incubated overnight at 4°C, and the unbound protein was washed off with 0.01 M  $\text{NaHCO}_3$  pH 8.3 buffer at room temperature. The bound antibody was eluted with 0.1 M  $\text{NaHCO}_3$  buffer, pH 8.0, containing 0.5 M NaCl, and the protein-containing fractions were tested by enzyme immunoassay (EIA) to confirm the presence of AGE-LDL antibodies. A second elution of the AGE-Sepharose column with 0.2 M glycine-HCl, pH 2.3, did not yield additional proteins, but was always carried out to regenerate the column.

## Characterization of the purified human AGE-LDL antibodies

**EIA.** AGE-LDL antibody activity was measured by a modification of the EIA originally described for the measurement of oxLDL antibodies (23, 26). Immulon type 1 plates (Dynatech Laboratories Inc, Chantilly, VA) were coated with 7.5  $\mu\text{g}$  of AGE-LDL/well. Peroxidase-labeled rabbit anti-human IgG (light and heavy chain specific, ICN Biochemicals Inc, Costa Mesa, CA) was used to detect captured antibodies. The difference between the optical density (OD) measured at 414 nm with unabsorbed and adsorbed samples was considered as reflecting antibody concentration, expressed in OD Units.

**Antibody avidity.** An estimate of the avidity of each purified antibody was obtained through the measurement of  $K_d$  by competitive enzyme immunoassay using a modification of Friguet's method (27), as adapted to the characterization of oxLDL antibodies by Mironova et al. (19). Flat-bottomed Immulon Type I plates were coated with 7.5  $\mu\text{g}$  of AGE-LDL per well. Purified AGE-LDL antibody was used at a final concentration of 100  $\mu\text{g}/\text{ml}$ . A series of antibody aliquots was absorbed using concentrations of AGE-LDL ranging from  $7.36 \times 10^{-7}$  to  $1.15 \times 10^{-8}$  M. The concentrations of antigen along with the absorbance values measured in unabsorbed and adsorbed samples were used to construct a plot of  $v/a$  versus  $v$  where  $v$  corresponds to bound antibody and  $v/a$  to bound antibody/free antigen at equilibrium (27). The slope of the plot was used to calculate the  $K_d$ .

**Immunoglobulin isotypes.** The distribution of immunoglobulin isotypes in the fractions eluted from the AGE-LDL column was determined by measuring IgG (total and subclasses 1, 2, 3, and 4), IgM, and IgA by radial immunodiffusion (RID) using low-level RID kits purchased from The Binding Site Inc., San Diego, CA.

**Cross-reactivity.** To determine whether AGE-LDL antibodies cross-reacted with oxLDL or native LDL, we performed EIA in which the plates were coated with AGE-LDL, and 200  $\mu\text{l}$  aliquots of purified antibodies were absorbed with 200  $\mu\text{l}$  of AGE-LDL, oxLDL, or native LDL at a concentration of 200  $\mu\text{g}/\text{ml}$ . An identical volume of buffer was added to an unabsorbed aliquot of the same antibodies. To determine whether unrelated serum proteins share the CML epitope(s) of LDL, we prepared CML-modified LDL and human serum albumin (HSA) at similar levels of modification, (5 mmol CML/mol lysine for LDL and 4 mmol CML/mol lysine for HSA). Each of these preparations (80  $\mu\text{g}$ ), as well as identical concentrations of the unmodified counterparts and of a preparation of AGE-LDL prepared as described earlier, were added to dilutions of four different purified AGE-LDL antibodies. The antibody dilutions were adjusted so that the OD (414 nm) of the unabsorbed samples reacting with AGE-LDL in the EIA varied between 0.6 and 1.0. The differences in OD measured with unabsorbed and adsorbed aliquots by EIA were considered indicative of the reactivity of the antibodies with the different modified LDL preparations.

## Isolation of LDL and IgG precipitated with 3.5% PEG

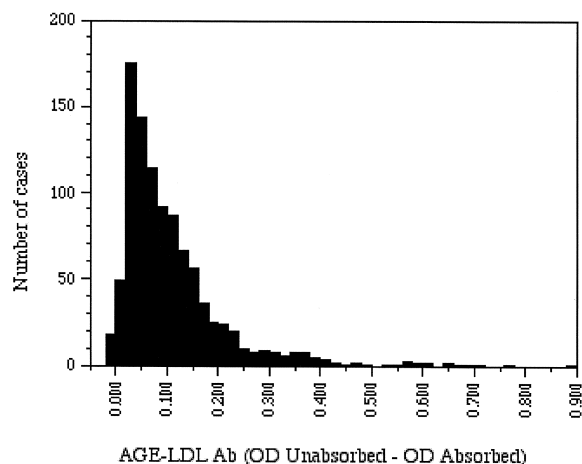
To better define the nature of LDL modifications responsible for the immune response that results in the formation of LDL-IC, we characterized the modifications of LDL precipitated as LDL-IC with 3.5% PEG from the serum of seven diabetic patients (20, 28). The sera were selected based on the previous detection of cholesterol and apolipoprotein B (apoB) in 3.5% PEG precipitates (29). To isolate the LDL moiety of LDL-IC, we precipitated serum aliquots with 3.5% PEG and fractionated the resuspended precipitates by affinity chromatography on Protein G-Sepharose (Amersham-Pharmacia). The column was washed with 0.1 M sodium phosphate buffer, pH 8.3, containing 0.5 M NaCl. Under these conditions, IgG antibodies of all specificities and subclasses

remained bound to the column while non-IgG antibodies and antigens (including lipoproteins) were contained in the wash-out. The wash-out was next fractionated by affinity chromatography on a heparin-agarose column (Sigma-Aldrich Corp., St. Louis, MO) that retained apoB-containing lipoproteins, while all other antigens and antibodies were eliminated in the flow-through fraction. The lipoprotein-containing samples were pooled and dialyzed against saline containing 0.3 mM EDTA, pH 8.0. Protein content was measured by the Lowry reaction (25) and AGE-ALE content quantified by SIM-GC-MS as described above.

## RESULTS

AGE-LDL antibodies were isolated from the sera of seven patients with Type 1 diabetes mellitus. These patients were selected from a group of 1,026 EDIC patients screened for AGE-LDL antibodies (Fig. 1). In the total population, the median concentration of AGE-LDL antibody was 0.211 OD units (range: 0 to 0.898). The patients were selected based on their high concentration of AGE-LDL antibodies and sample availability. The median concentration of AGE-LDL antibody in the samples chosen for isolation of AGE-LDL antibodies was 0.588 OD units (range: 0.477–0.761). Table 1 shows that the purified AGE-LDL antibodies were predominantly of the IgG isotype, more specifically of the IgG1 and IgG3 subclasses. In some sera, IgM antibodies were detected in relatively large concentrations, although always lower than those of IgG antibodies. The  $K_d$  values varied between 0.76 to  $8.1 \times 10^{-9}$  mol/l (average  $4.76 \pm 2.52 \times 10^{-9}$  mol/l).

The AGE-LDL preparation used to prepare the affinity chromatography column used to purify AGE-LDL antibodies contained primarily CML (14.6 mmol/mol lysine) and smaller amounts of CEL (2.7 mmol/mol lysine), but no detectable MDA- or HNE-lysine (Table 2). The results suggest that under the conditions used in our laboratory to prepare AGE-modified LDL, the two major glycoxidation products, CML and CEL, were formed in high amounts



**Fig. 1.** Distribution of advanced glycosylation end-products (AGE)-LDL antibody levels in 1,026 blood samples studied as part of the Epidemiology of Diabetes Complication (EDIC) protocol.

TABLE 1. Characteristics of AGE-LDL antibodies isolated from the sera of seven patients with Type 1 diabetes by affinity chromatography on AGE-LDL-Sepharose columns

Sample	Antibody Isotypes							$K_d (\times 10^{-9})$
	Total IgG	IgG1	IgG2	IgG3	IgG4	IgM	IgA	
	<i>mg/l</i>							
A	23.2	23.2	nd	nd	nd	12.9	1.6	4.80
B	52.5	28.1	9.9	14.5	nd	4.8	0.9	6.80
C	32.2	16.1	nd	6.1	nd	9.5	0.6	8.09
D	48.9	23.2	7.5	18.2	nd	12.9	3.5	0.76
E	38.9	20.7	nd	18.2	nd	26.6	2.2	4.52
F	26.0	11.5	nd	14.5	nd	11.2	4.3	2.43
G	92.6	58.6	4.0	30.0	nd	17.5	5.4	5.92

The levels of the four IgG subclasses, IgA, and IgM were determined by radial immunodiffusion. The total IgG concentration was calculated by adding the concentrations of the four IgG subclasses. Dissociation constants ( $K_d$ ) were determined by enzymeimmunoassay (EIA). AGE, advanced glycosylation end-product; nd, not detectable.

and there was minimal lipid oxidation. Therefore, the purified AGE-LDL antibodies should recognize CML and CEL epitopes.

To obtain a better understanding of the nature of LDL modifications recognized by human autoantibodies, we used an alternative strategy that consisted in isolating and characterizing the lipoproteins involved in spontaneous formation of IC. The analysis of the coprecipitated lipoproteins (Fig. 2) showed the presence of CML, CEL, and MDA-lysine, suggesting that a mixture of antibodies to oxidized lipoproteins, recognizing MDA-lysine epitopes, and to AGE-modified lipoproteins, recognizing CML and CEL modifications, must have been involved in IC formation.

Finally, we carried out two studies to define the specificity of our purified AGE-LDL antibodies. In the first study (Fig. 3A), we compared the reduction in reactivity of purified AGE antibodies with AGE-LDL caused by preincubation with AGE-LDL, oxLDL, and native (unmodified) LDL. The preincubation with AGE-LDL resulted in a significantly higher reduction in reactivity with AGE-LDL than the preincubation with either native or oxLDL. To determine whether CML was a major epitope recognized by AGE-LDL antibodies and whether the epitopes associated with CML are identical in different proteins, we compared the reduction in reactivity with AGE-LDL of four different AGE-LDL antibodies caused by preincubation with AGE-LDL, CML-LDL, and CML-HSA (Fig. 3B). The results showed that the reductions in reactivity caused by

TABLE 2. Advanced glycosylation and advanced lipoxidation end-products content of AGE-LDL preparation utilized for the purification of AGE-LDL antibodies and for the assay of AGE-LDL antibodies by EIA

Protein Preparation	CML	CEL	MDA-Lysine	HNE-Lysine
AGE-LDL (n = 2)	13.8–15.5 <sup>a</sup>	2.7–2.7 <sup>b</sup>	nd	nd
Native LDL (n = 3)	0.13–0.16	0.03–0.04	nd	nd

CML, (carboxymethyl)lysine; CEL, (carboxyethyl)lysine; MDA, malondialdehyde; HNE, 4-hydroxynonenal.

<sup>a</sup> Values expressed in mmol/mol lysine.

<sup>b</sup> Data are range of values for indicated number of samples.

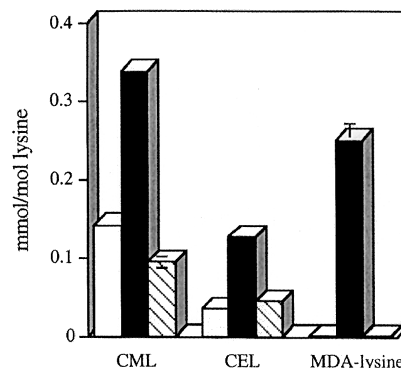


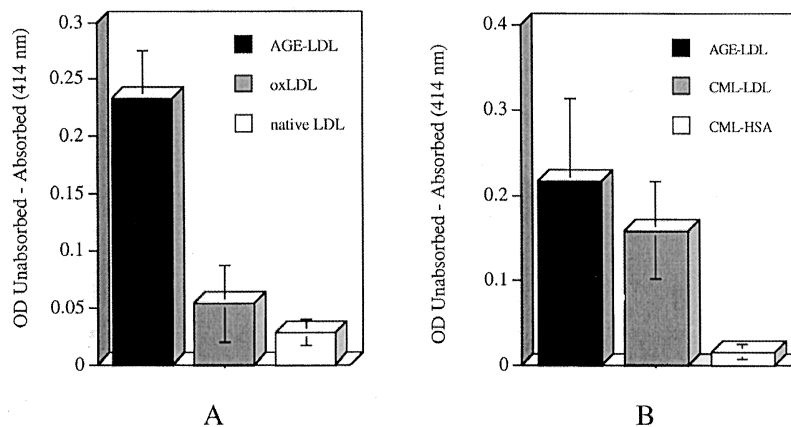
Fig. 2. AGE-advanced lipoxidation end-products (ALE) content of apoB-containing lipoproteins isolated from polyethelene glycol (PEG)-precipitated immune complexes (ICs). Masked samples of bound (closed bars) and unbound (hatched bars) fractions from the heparin-agarose column, as well as native LDL (open bars), were analyzed by selected ion-monitoring gas chromatography/mass spectrometry (SIM-GC-MS) for their content of (carboxymethyl)lysine (CML), (carboxyethyl)lysine (CEL), and malondialdehyde (MDA)-lysine; 4-hydroxynonenal (HNE)-lysine ions were monitored but not detected in any samples. The values are expressed as mean  $\pm$  SEM (n = 6).

preincubation with AGE-LDL or CML-LDL were not significantly different, while a significant difference was observed between samples preincubated with CML-LDL and CML-HSA, suggesting that the CML-lysine epitopes are not identical in unrelated proteins.

## DISCUSSION

The pathogenesis of macrovascular disease in diabetes mellitus has received considerable attention as a consequence of the increased incidence of macrovascular complications in diabetic patients, including coronary heart disease, cerebrovascular disease, and peripheral vascular disease (30). In recent years, atherosclerosis has been redefined as a chronic inflammatory process (31). The definition of factors that may trigger the inflammatory process is of obvious importance, and immunologic mechanisms, both cell mediated (32–34) and antibody mediated (12, 15, 35, 36), have been proposed as playing that role. The involvement of antigen-antibody complexes formed by modified lipoproteins and corresponding antibodies in the pathogenesis of atherosclerosis is supported by the fact that those complexes have both proinflammatory and atherogenic properties (16, 18, 37–39), and by epidemiological associations between immune complex levels and an increased risk for the development of coronary heart disease (13, 40).

Diabetic patients would appear to be at particular risk for the development of IC involving modified lipoproteins because chronic hyperglycemia leads to protein glycosylation. Glycosylated proteins, including LDL, are more susceptible to oxidation (41, 42). The synergy of glycosylation and oxidation results in the formation of AGE or glycoxidation products such as CML and CEL (43, 44).



**Fig. 3.** Specificity of purified AGE-LDL antibodies. In A, we compare the reduction in reactivity with AGE-LDL of six different antibodies absorbed with AGE-LDL, oxidized LDL (oxLDL), and native (unmodified) LDL. The differences in reactivity after absorption with AGE-LDL were significantly different from those observed after absorption with oxLDL ( $P = 0.009$ ) and with native LDL ( $P = 0.006$ ) by the unpaired Student's *t*-test with Welch correction. B: Shows the extent of cross-reactivity of four different AGE-LDL antibodies with two different CML-modified proteins. The degree of modification of LDL and HSA was similar (5 mmol CML/mol lysine for LDL and 4 mmol CML/mol lysine for HSA). The differences in reactivity after absorption with AGE-LDL and CML-LDL were not significantly different ( $P = 0.886$ ), while the differences in reactivity after absorption with CML-LDL and CML-HSA were significantly different ( $P = 0.029$ , Mann-Whitney test). The values are expressed as the mean  $\pm$  SEM.

Both oxLDL and AGE-modified proteins have been proven to be immunogenic (45–47), and autoantibodies to oxLDL and to AGE-modified proteins have been described (10, 48). The existence of circulating ICs containing AGE-modified proteins was also recently reported by Turk et al. (10). However, the evidence for the existence of antibodies to AGE-modified proteins and ICs formed by AGE-modified proteins and the corresponding antibodies is based on the use of AGE-albumin. No direct evidence for the existence of autoantibodies reacting with AGE-modified lipoproteins has been published.

The AGE-LDL autoantibodies we have isolated from the sera of patients with diabetes mellitus are remarkable for their relative homogeneity in isotype distribution and avidity. The predominance of IgG1 and IgG3 is similar to what had been previously observed with isolated oxLDL antibodies, but the average avidity of AGE-LDL antibodies is higher than that of oxLDL antibodies. This is an important observation, because it implies that AGE-LDL antibodies have the required characteristics to form stable IC likely to interact with and activate inflammatory cells (22).

To define the immunogenic modifications associated with AGE-LDL formation, we used several different approaches. The identification of CML and CEL as major modifications in the AGE preparations used to prepare the AGE-LDL immunoabsorbant columns suggested that the autoantibodies would recognize CML and CEL epitopes. However, other important epitopes may not have been present in AGE-LDL prepared in vitro. To further clarify this issue, we isolated apoB-rich lipoproteins (including LDL) from the supernatant and precipitates obtained after incubation of sera from diabetic subjects with 3.5% PEG by affinity chromatography in heparin-Sepharose. This concentration of PEG is known to precipitate IC but not soluble antigens or antibodies (49, 50).

We have also established that soluble, native LDL is only precipitated in vestigial amounts by 3.5% PEG (20). The finding of an enrichment of CML, CEL, and MDA-lysine in the precipitated lipoproteins allows two conclusions. First, it supports the data obtained in the analysis of the AGE-LDL used to prepare the immunoabsorbant pointing to CML and CEL as the major modifications recognized by AGE-LDL autoantibodies. Second, it suggests that diabetics have multiple autoantibodies to different forms of modified LDL, including AGE-LDL and oxLDL. The residual reactivity of purified AGE-LDL antibodies with nLDL may be explained by the presence of spontaneously formed AGE-LDL in LDL isolated from normal donors, while the reactivity with oxLDL is likely to result from the generation of CML-lysine during copper oxidation. Both possibilities are supported by findings reported in a previous report of our studies on the isolation and characterization of oxLDL antibodies. Freshly isolated LDL was found to contain measurable concentrations of CML-lysine, which increased 3- to 15-fold after copper oxidation for 19 h (23).

One question that remained to be answered was whether antibodies to CML-modified proteins are able to recognize any type of AGE-modified protein. Crystallographic studies of defined antigen-antibody complexes suggest that that would not be the case, because even if the CML group is the immunodominant structure recognized by AGE-protein antibodies, the epitopes are defined not only by the immunodominant determinant, but also by the 15–22 amino acids in its immediate vicinity (51). The likelihood that these amino acids would be distributed and folded in identical spatial arrangements in proteins as different as albumin and apoB appears highly

unlikely. The fact that preparations of CML-LDL were able to decrease the reactivity of purified AGE-LDL antibodies, but identically modified CML-albumin preparations were ineffective, proves that AGE-protein antibodies are specific for different AGE-modified proteins. As such, the detection of AGE antibodies using AGE-modified albumin as substrate does not prove that antibodies to AGE-LDL are present in the same patient. This is important in the context of the relationship of AGE antibodies and macrovascular disease, because we have proven in previous studies that IC involving proteins other than LDL do not share the atherogenic properties of LDL-IC (16). ■■

## APPENDIX

### Participants of the DCCT/EDIC Research Group

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