*Short communication***Antibodies against advanced glycation end product N^{ϵ} -(carboxymethyl)lysine in healthy controls and diabetic patients****D. Vay¹, M. Vidali¹, G. Allochis², C. Cusaro², R. Rolla¹, E. Mottaran¹, G. Bellomo¹, E. Albano¹**¹ Dept. of Medical Sciences, University “Amedeo Avogadro” of East Piedmont, Novara, Italy² Unit of Diabetology and Metabolic Diseases, Hospital Maggiore e della Carità, Novara, Italy**Abstract**

Aims/hypothesis. N^{ϵ} -(carboxymethyl)lysine (CML) is one of the end products of protein glycoxidation and its accumulation is associated with diabetes complications. Since CML-modified proteins are immunogenic, we have investigated the presence of anti-CML antibodies in diabetic patients.

Methods. Antibodies against CML-modified human serum albumin (HSA) were measured by direct enzyme-linked immunosorbent assay in the sera from 289 non-selected diabetic and in 120 healthy control subjects.

Results. Immunoglobulin-G reactivity towards CML-HSA was significantly higher in diabetic than in control sera. The presence of anti-CML IgG in diabetics, however, was not influenced by age, duration of disease or glycaemic control. Analysis of distribution

frequency revealed that anti-CML IgG in both control and diabetic subjects were not normally distributed and that the distribution curves were similar in the two groups. Moreover, only 14 % of the diabetic subjects displayed antibody binding to CML-HSA above 95 centile in the control cohort. Competition experiments confirmed that the IgG detected in both control and diabetic groups were specific for CML epitopes and did not recognise glycated-HSA in which CML formation was inhibited.

Conclusion/interpretation. The presence of anti-CML IgG in diabetic sera is probably not related to the development of an immune response against protein glycoxidation products. [Diabetologia (2000) 43: 1385–1388]

Keywords AGEs, glycoxidation, diabetes, autoantibodies.

N^{ϵ} -(carboxymethyl)lysine (CML) is one of the best characterised advanced glycation end products (AGEs) in protein [1]. A number of studies have shown that tissue accumulation of CML correlates with the severity of diabetic complications [2, 3]. Proteins containing AGEs are highly immunogenic and CML is one of the major epitopes recognised by anti-AGE antibodies [4, 5]. The presence of anti-

CML antibodies in the serum of streptozotocin-diabetic rats as well as in a small number of diabetic patients have been reported recently [6]. Considering the potential use of anti-CML antibodies as a marker of AGEs deposition during diabetes, we have investigated the presence of anti-CML antibodies in a large group of non-selected diabetic patients from whom samples of serum were taken periodically for laboratory analysis.

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Abbreviations: AGEs, Advanced glycation end products; CML, N^{ϵ} -(carboxymethyl)lysine; CEL, N^{ϵ} -(carboxyethyl)lysine; DTPA, diethylenetriaminepentacetic acid; HSA, human serum albumin; OD, optical density.

Materials and methods

We have investigated 154 male and 135 female diabetic patients (14 with Type I (insulin-dependent) diabetes mellitus and 275 with Type II (non-insulin-dependent) diabetes mellitus; mean duration of diabetes 10 ± 7.7 years; mean age

66 ± 11) undergoing periodic checks at the Diabetes Care Unit of the Hospital Maggiore della Carità (Novara, Italy) during a two week period. Abnormal fructosamine and glycated haemoglobin values were present in 47% and 7% of the patients, respectively. Microalbuminuria was detectable in 65 patients, whereas macroalbuminuria was present in 20 patients.

Blood donors (120; 100 male and 20 female; mean age 42 ± 11) were enrolled as controls in the same two week period when the diabetic patients were checked. All subjects gave informed consent to the analysis and the study was planned according to the guide-lines of our University ethics committee.

Antigen preparation. Adducts of CML-protein were generated by a 24 h reaction of human serum albumin (HSA) with glyoxylic acid under reducing conditions as described previously [4]. Glycated HSA poor in CML was prepared according to a previous study [7] using diethylene-triaminepentacetic acid (DTPA) as a chelating agent. The fructoselysine content in glycated-HSA was 131 µmol/g of protein. We obtained *N*^ε-(carboxyethyl)lysine-modified HSA by reacting HSA with 100 mmol/l methylglyoxal as reported previously [8]. For the preparation of CML-containing beads 10 ml of L-lysine-Sepharose 4B beads suspension in 0.2 mol/l Na-phosphate buffer pH 7.4, (equivalent to 0.8 mmol/l lysine) were derived by a 24 h reaction at 37°C with 45 mmol/l glyoxylic acid and 150 mmol/l NaCNBH₃. At the end of incubation the beads were washed three times with 0.2 mol/l Na-phosphate buffer pH 7.4 and were re-suspended in 10 ml of the same buffer. Amino group derivation in Sepharose-lysine beads accounted for 52%.

Measurement of antibody titers. Polystyrene microwell plates for enzyme-linked immunosorbent assay (ELISA) (Immulon IV; Nunc, Fisher Scientific St. Louis Mo., USA) were coated for 4 h at 37°C with 0.05 mg/ml of modified or native HSA solubilised in 0.1 mol/l bicarbonate buffer pH 9.6. Non-specific binding sites were blocked by 1 h incubation with coating buffer containing 3% bovine serum albumin in phosphate buffered saline (PBS) pH 7.4. The coated wells were washed three times with PBS containing 0.25% Triton X-100 and the sera (1:50 dilution in coating buffer) were added in duplicate. After 1 h incubation at 37°C the plates were washed three times with PBS-0.25% Triton X-100 and peroxidase-linked goat anti-human IgG (dilution 1:6000), IgA (dilution 1:9000) or IgM (dilution 1:5000) (Dako, Milan, Italy) were added and incubated for 60 min at 37°C. Antibody binding was revealed colorimetrically at 490 nm by adding a reaction mixture containing 0.4 mg/ml of 1-phenyldiamine, 0.4 µl/ml hydrogen peroxide (30%), 5.1 mg/ml citric acid, 6.1 mg/ml anhydrous Na₂HPO₄, pH 5.0.

Data analysis and statistical calculations. Statistical analyses were done using Mann-Whitney test. Correlations were estimated by Spearman's *r* values. Preliminary evaluations of the groups considered for normality distribution were done by Kolmogorov and Smirnov test. We considered differences significant at *p* less than 0.05.

Results

We observed that control and diabetic subjects did not show a statistically significant difference in the reactivity towards unmodified HSA [optical density (OD) 0.066 ± 0.018 versus 0.072 ± 0.029]. Conversely, an appreciable IgG binding to CML-HSA was evi-

Fig. 1. Frequency-distribution profile and mean values of IgG reactivity towards *N*^ε-(carboxymethyl)lysine-modified HSA (CML-HSA) in the sera from 289 diabetic patients (■) and 120 healthy control subjects (▨). HSA was modified by 24 h reaction at 37°C with 0.75 mol/l glyoxylic acid and 0.3 mol/l NaCNBH₃ [4]. Human sera were tested at 1:50 dilution in microplate ELISA plates coated with CML-HSA or with unmodified HSA and the IgG binding was shown with peroxidase-linked goat anti-human IgG anti-serum. The results are expressed as optical densities at 490 nm after subtracting the contribution from the individual reactivity with unmodified HSA. Minimum and maximum OD values in each group were as follows: Controls: OD 0–1.245; Diabetics: OD 0–2.458. Statistical significance: *p* < 0.0001 vs control subjects

dent in both groups (OD 0.190 ± 0.162 and 0.260 ± 0.321, respectively). No reaction was detectable in the IgA and IgM fractions (data not shown). Immunoglobulin-G binding to CML-HSA in control and diabetic subjects was not significantly different between men and women and neither was dependent on age (data not shown). Taking this into account, we calculated the specific anti-CML reactivity, as the difference between IgG binding to CML-HSA and native HSA. Anti-CML IgG were significantly (*p* < 0.0001) higher in diabetic than in control subjects (Fig. 1). Anti-CML reactivity was also not influenced by the duration of diabetes or by the presence of diabetic nephropathy, as indicated by either microalbuminuria or macroalbuminuria (data not shown). Furthermore, IgG against CML-HSA in diabetics with abnormal fructosamine (OD 0.198 ± 0.322) were not statistically significantly different from those in the patients with good metabolic control (OD 0.179 ± 0.301). No association was observed between glycated haemoglobin values and anti-CML reactivity (data not shown).

Frequency distribution analysis showed that anti-CML reactivity in both diabetic and control subjects

A Lysine-Sepharose bead suspension (μl)

B Control subjects Diabetic subjects

Fig. 2. Effect of pre-adsorption with CML-containing Sepharose beads on the binding of IgG from diabetic patients or healthy subjects to CML-modified human serum albumin (CML-HSA). Beads containing CML were prepared as reported in the methods section. Pre-adsorption experiments were done by incubating overnight at 4°C human sera (1:50 dilution) with CML-containing lysine-Sepharose L4 beads. Similar amounts of non-modified lysine-Sepharose beads were used as control. At the end of the incubation the beads were removed by centrifugation. Aliquots of the pre-adsorbed sera were then added in, duplicated to ELISA plates coated with either CML-HSA or native HSA and antibody binding was assayed as described in the methods section. **A** shows pre-adsorption experiments done using increasing amounts of CML-modified (filled symbols) and unmodified (open symbols) beads. **B** shows the effect of pre-adsorption (■) with 300 μl of CML-containing beads suspension (equivalent to 120 $\mu\text{mol/l}$ of CML residues) of 10 control and 20 diabetic sera with different anti-CML reactivity. As a reference the ELISA readings in non-preadsorbed (▨) sera is shown. The antibody binding inhibited by CML-containing beads in the different groups was calculated by subtracting the optical density values following pre-adsorption of the same sera with CML-modified and non-modified beads, respectively. The results are expressed as per cent of the optical densities at 490 nm in non pre-adsorbed sera

was not normally distributed and that the distribution curves in both groups were similar (Fig. 1). Taking the OD values within 95 centile in the control group (OD 0.354) we observed that only 41 out of 289 diabetic patients (14%) displayed antibody binding to CML-HSA above this threshold (Fig. 1). Titration experiments using control and diabetic sera with high anti-CML reactivity also did not show appreciable differences in CML-HSA recognition (data not shown). Neither control nor diabetic sera showed appreciable binding to glycated-HSA in which oxidative CML formation was blocked by adding the iron chelating agent DTPA (data not shown). Sera with high reactivity towards CML-HSA ($n = 26$) recognised to some extent N^{ϵ} -(carboxyethyl)lysine (CEL)-containing HSA; however, the IgG binding to this latter epitope was about fourfold lower than that of CML (OD 0.236 ± 0.400 vs OD 1.074 ± 0.518 , respectively; $p < 0.0001$). The specificity of the antibodies for CML epitopes was confirmed by pre-adsorbing the sera with CML-coated Sepharose beads prepared by reacting Sepharose-lysine with glyoxylic acid under reducing conditions. Overnight incubation of four CML-reactive diabetic sera in the presence of increasing amounts of CML-containing Sepharose beads caused a dose-dependent decrease of IgG binding to CML-HSA, whereas a similar incubation with unmodified lysine-Sepharose beads had no effect (Fig. 2). In further experiments the pre-adsorption of CML-reactive sera from 10 control and 20 diabetic subjects with CML-containing beads (equivalent to 120 $\mu\text{mol/l}$ of CML residues) reduced by 88% the antibody binding to CML-HSA (Fig. 2). A similar inhibition (about 70%) was also observed when CML-preadsorbed sera were tested with CEL-HSA (data not shown).

Discussion

The presence of increased levels of antibodies recognising AGE-modified and CML-modified proteins in the serum of streptozotocin-diabetic rats have been shown recently [6]. The isolation of IgG with similar specificity from the plasma of diabetic patients was also reported [6]. In particular, significantly higher levels of these antibodies in patients with renal failure caused by diabetes or non-diabetic pathologies as compared to healthy controls or diabetics without renal failure was observed [6]. In this study, however, only ten patients in each experimental group were investigated. Our results obtained by analysing a large group of non-selected diabetic patients compared with normoglycaemic subjects who donated blood confirmed that anti-CML IgG are statistically significantly increased in diabetic subjects. Nonetheless, the anti-CML activity in these patients seems unrelated with either the duration of the dis-

ease or with its metabolic control. We have observed that about 36 % of the control subjects have appreciable titres of anti-CML IgG. Titration curves and competition experiments confirm that in both control and diabetic groups anti-CML reactivity is specific for this epitope and is probably mediated by high affinity IgG.

The degradation of Amadori adducts resulting from glucose binding to proteins is considered to be the main pathway for CML formation *in vivo* [1]. No binding to glycated-HSA has, however, been observed when CML formation through metal-catalysed oxidative degradation of Amadori products is inhibited by iron-chelation [7]. Sera with high anti-CML reactivity recognise, to a lesser extent, branched *N*^ε-(carboxyethyl)-lysine adducts resulting from methylglyoxal reaction with HSA [8]. The sera preadsorption with CML-Sepharose greatly reduced such a reactivity, indicating that anti-CML-IgG can cross-react with structurally related epitopes.

Several lines of evidence indicate that tissue accumulation of advanced glycoxidation end products (AGEs) can play a part in the development of diabetes complications [2, 3]. The analysis of the frequency distribution profile shows that only 14 % of the diabetic subjects display antibody binding to CML-HSA above the 95 centile of the control group. Moreover, anti-CML immune reactivity in diabetic patients seems unrelated to renal complications. These findings suggest that immunisation against CML antigens does not account for the presence of these antibodies. Recent studies have shown that CML residues increase in human tissues during senescence [9]. Moreover, CML can also originate during lipid peroxidation reactions [10]. Thus, it is possible that anti-CML IgG might represent naturally-occurring antibodies involved in the scavenging of aged or of oxidised biological structures.

In conclusion anti-CML IgG are detectable in an appreciable number of either control or diabetic subjects but their presence is unrelated to an immune response induced by AGE accumulation.

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