Anti-Modified LDL Antibodies, LDL-Containing Immune Complexes, and Susceptibility of LDL to In Vitro Oxidation in Patients With Type 2 Diabetes

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We investigated the hypothesis that modified lipoproteins trigger an immune response leading to the production of autoantibodies and subsequently to the formation of atherogenic immune complexes (IC). We recruited 20 type 2 diabetic patients with macrovascular disease, 14 nondiabetic patients with coronary artery disease (CAD), and 34 healthy control subjects matched for age, sex, and race. Serum antibodies to oxidized and glycated LDL did not differ significantly among the 3 groups. Serum IC contained variable, but not statistically different, amounts of IgG, IgM, and IgA. In contrast, the content of cholesterol in IC isolated from diabetic patients was significantly higher than that in IC isolated from control subjects, and the content of apolipoprotein (apo)-B was significantly higher than that in IC isolated from control subjects and patients with CAD. Cholesteryl ester accumulation in human monocyte-derived macrophages incubated with IC, a measure of the atherogenic potential of IC, was significantly higher in macrophages incubated with red blood cell–adsorbed IC isolated from diabetic patients compared with IC isolated from control subjects ($P < 0.03$) or from patients with CAD ($P < 0.04$) and was strongly correlated with the content of apoB ($r = 0.68$, $P < 0.001$) and cholesterol ($r = 0.61$, $P < 0.001$) in IC. LDL from diabetic patients was more susceptible to oxidation in vitro, was significantly smaller, and contained significantly less α-tocopherol than LDL isolated from subjects in the other groups. In addition, the n-3 polyunsaturated fatty acid content of phospholipids and cholesteryl esters in LDL isolated from diabetic patients was significantly increased ($P < 0.05$) compared with that from patients with CAD or from control subjects. We postulate that LDL size, susceptibility to oxidation, and lipid fatty acid composition may play a critical role in the production of antibodies to oxidized LDL and consequently in the formation of LDL-containing IC in patients with type 2 diabetes. *Diabetes* 49:1033–1041, 2000

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apo, apolipoprotein; CAD, coronary artery disease; CE, cholesteryl ester; gly-LDL, glycated LDL; IC, immune complexes; LDL-IC, LDL-containing immune complexes; OD, optical density; ox-LDL, oxidized LDL; PEG, polyethylene glycol; PEG-IC, polyethylene glycol–precipitated immune complexes; RBC, red blood cell; RBC-IC, immune complexes adsorbed to red blood cells.

It is well known that diabetes is associated with an increased incidence of macrovascular complications, including coronary artery disease (CAD) and cerebrovascular and peripheral vascular disease (1). The mechanisms by which diabetes accelerates atherosclerosis are not well understood. The customary clusters of risk factors for CAD, which are more common in patients with diabetes, are not sufficient to explain this phenomenon. In recent years, it was proposed that an increased level of modified lipoproteins might be an additional factor contributing to the accelerated development of macrovascular complications in diabetes (2). These modified lipoproteins are thought to contribute to the development of atherosclerosis by several mechanisms. One is their ability to induce intracellular accumulation of cholesteryl esters (CEs) in macrophages, which leads to their transformation into foam cells after being taken up by the scavenger receptor pathway (3,4). More recently, it was proposed that modified lipoproteins might contribute to atherogenesis by another mechanism—their ability to trigger an immune response leading to the production of autoantibodies and subsequently to the formation of immune complexes (IC) (5). Studies carried out in our laboratory (6,7) showed that incubation of human monocyte-derived macrophages with insoluble LDL-containing IC (LDL-IC) prepared with human LDL and rabbit anti-LDL antibodies induces foam cell formation in vitro more efficiently than any other known mechanism. Similar results were obtained with soluble IC prepared in antigen excess and presented to macrophages after absorption to red blood cells (RBCs) (8,9). Studies published by Tertov et al. (10,11) and Orekhov et al. (12) supported the pathogenic role of LDL-IC in vivo. These authors showed that LDL-IC was present at higher levels in the sera of patients with CAD than in normal individuals (10,11) and that the cholesterol content of LDL-IC is a powerful discriminator for the presence of coronary atherosclerosis (12).

Most of the published data concerning the relationship between IC and atherosclerosis, however, has been obtained in nondiabetic subjects. Because protein glycation and glucose autoxidation, common events occurring in diabetic patients, are known to generate free radicals and thus may catalyze lipid peroxidation (2,13,14), it seemed likely that diabetic patients would be prone to develop antibodies to modified

lipoproteins, which could lead to the subsequent formation of IC. Recently, we have reported (15) that atherogenic IC are present in the sera of type 1 diabetic patients without macrovascular complications as well as in the sera of normal individuals. These IC contain anti–oxidized (ox)-LDL antibodies, and their atherogenicity, i.e., their ability to induce CE accumulation by human macrophages, is mostly dependent on their cholesterol and apolipoprotein (apo)-B content. Furthermore, we have demonstrated that the content of apoB in IC isolated from the patients with type 1 diabetes was a powerful predictor of macrovascular complications in these patients (16). Our present study was conducted to determine if IC containing oxidized LDL are associated with the presence of macrovascular complications in patients with type 2 diabetes. We also studied the composition of LDL and susceptibility of LDL to in vitro oxidation, the factors that may influence LDL modification and, thus, facilitate the production of anti–modified LDL antibodies and lead to the formation of IC.

RESEARCH DESIGN AND METHODS

Patients. We studied 20 type 2 diabetes patients with documented macrovascular disease, 14 nondiabetic patients with CAD, and 34 healthy control subjects matched for age, sex, and race. The age of patients with diabetes, patients with CAD, and normal individuals averaged 59.1 ± 1.3, 56.9 ± 2.6, and 58.4 ± 1.6 years, respectively. The mean duration of diabetes was 18.0 ± 2.2 years. Macrovascular disease was diagnosed on the basis of clinical evidence of CAD, cerebrovascular or peripheral vascular disease, and documented history of myocardial infarction, stroke, bypass surgery, or angioplasty, and was confirmed by angiography. According to these criteria, 11 diabetic patients had CAD alone, 5 had CAD with peripheral vascular disease, 2 had CAD with cerebrovascular disease, and 2 had a combination of CAD with peripheral vascular disease and cerebrovascular disease. The presence of coronary atherosclerosis in all nondiabetic patients with CAD was diagnosed on the basis of clinical evidence of CAD and/or was confirmed by angiography. Exclusion criteria for each group of subjects were as follows: malignancy, untreated thyroid disease, alcohol excess, smoking, intercurrent illness, treatment with lipid-lowering drugs, or receiving antioxidant vitamin supplements. Patients with daily urinary protein excretion >0.5 g and serum creatinine >124 μmol/l or patients with liver enzymes >1.5 times the upper limit of normal were also excluded. Each patient with diabetes or CAD and his or her matched control subject were studied on the same day. Written informed consent was obtained from all subjects.

Clinical characteristics of patients with type 2 diabetes, patients with CAD, and control subjects are shown in Table 1. As expected, the diabetic patients had significantly higher levels of plasma glucose and HbA_{1c} than CAD patients and control subjects. Patients with type 2 diabetes were significantly heavier than normal control subjects. Diabetic patients had significantly higher levels of plasma triglycerides and VLDL cholesterol than CAD patients and control subjects. They also had significantly higher levels of plasma lipoprotein(a) than patients with CAD or control subjects. No difference was found in the level of plasma fibrinogen and serum uric acid in these groups.

Measurement of antibodies to modified LDL. Blood samples were drawn after an overnight fast. Serum was separated by centrifugation and used for measurement of antibody levels and precipitation of IC. Serum aliquots were stored at –70°C before being tested, and all tests were performed within 4 weeks of blood collection.

Antibodies to modified LDL were determined using the previously reported competitive enzymoimmunoassay for antibodies to oxidized LDL (17). For the assay of antibodies against glycated LDL (gly-LDL), oxidized LDL was replaced by identical concentrations of gly-LDL in the coating and absorption steps. Gly-LDL was prepared as previously described (18). The peroxidase-conjugated sheep anti-rabbit antibody used in all the assays was a polyvalent anti-IgG antibody that reacts with γ-heavy chains as well as with κ- and λ-chains; thus, it recognizes all immunoglobulin isotypes. The assays were calibrated using IgG purified from a rabbit anti-apoB serum, which reacted with normal and all forms of modified LDL used in our assay (17). The antibody concentrations were expressed in ngEq/ml and were calculated using the difference in optical density (OD) (414 nm) between unabsorbed sera and serum absorbed by either oxidized or glycated LDL used to coat the plate as the OD level (17).

Precipitation of soluble IC with polyethylene glycol. Two and a half milliliters of a freshly prepared solution containing 7% (wt/vol) polyethylene glycol (PEG) 6000 in borate buffered-saline, pH 8.4, sterilized by filtration through a 0.22-μm filter, was slowly added under constant mixing to 2.5 ml sera in a sterile 15-ml thick glass centrifuge tube. The samples, containing a final PEG concentration of 3.5%, were incubated overnight at 4°C and then centrifuged at 3,000 rpm for 20 min. The precipitates were washed once with 14 ml chilled 3.5% PEG in borate-buffered saline, centrifuged again, and gently resuspended in 2 ml Tyrode's solution (Ca^{2+}, Mg^{2+}) at 37°C.

Characterization of PEG-precipitated soluble IC. The protein content of PEG precipitates was measured using a modified Lowry assay (19). IgG, IgA, and IgM were determined using a commercially available radial immunodiffusion assay (Low Level RID plates; The Binding Site, San Diego, CA). The cholesterol content of IC was determined by extraction of the PEG precipitates with chloroform/methanol (2:1,vol/vol) and by measurement of total cholesterol by gas chromatography as described below for macrophage cell cultures. The concentration of apoB in IC was determined by quantitative immunoelectrophoresis as described previously (20).

Preparation of RBC-bound IC. RBCs were isolated from the blood of type O Rh^+ healthy volunteers after separation of the buffy coat by sedimentation in dextran as described previously (21). The RBCs to be used in subsequent experiments were washed 4 times with Tyrode's solution, pH 7.35, and counted in a Coulter Counter Model ZF (Coulter Electronics, Hialeah, FL). The contamination of the RBCs with leukocytes and platelets was minimal (0.02–0.03% leukocytes and 0.01–0.02% platelets).

Immune complexes adsorbed to RBCs (RBC-IC) were prepared as previously described (22). In short, 1×10^8 RBCs were incubated at 37°C for 1 h under constant mixing with 1.5 ml PEG-precipitated soluble IC (PEG-IC), prepared as described above. RBCs were washed once with Tyrode's solution to eliminate unabsorbed reactants, resuspended in 250 μl Iscove modified Dulbecco's medium, and γ-irradiated (3,000 rad/4.1 min) in Gammacel 1000 (Atomic Energy of Canada, Kanata, ON, Canada).

TABLE 1
Clinical characteristics of type 2 diabetic and CAD patients and control subjects

Parameter	Type 2 diabetic patients	CAD patients	Control subjects
n (M/F)	20 (18/2)	14 (14/0)	34 (32/2)
Age (years)	59.1 ± 1.3	56.9 ± 2.6	58.4 ± 1.6
BMI (kg/m^2)	30.38 ± 0.80*	27.40 ± 1.18	26.73 ± 0.39
Glucose (mmol/l)	10.0 ± 1.1†	4.6 ± 0.4	4.7 ± 0.1
HbA_{1c} (%)	7.8 ± 0.6†	5.3 ± 0.3	5.1 ± 0.2
Total plasma cholesterol (mmol/l)	6.26 ± 0.47	5.35 ± 0.41	5.30 ± 0.13
Total plasma triglyceride (mmol/l)	4.25 ± 1.08†	1.76 ± 0.23	1.76 ± 0.20
VLDL cholesterol (mmol/l)	1.78 ± 0.49†	0.8 ± 0.1	0.8 ± 0.1
LDL cholesterol (mmol/l)	3.26 ± 0.28	3.57± 0.36	3.39 ± 0.16
HDL cholesterol (mmol/l)	0.93 ± 0.08	1.0 ± 0.08	1.11 ± 0.08
Plasma Lp(a) (mg/dl)	27.9 ± 4.9†	16.2 ± 4.2	16.9 ± 3.0
Serum uric acid (mmol/l)	0.36 ± 0.02	0.38 ± 0.02	0.36 ± 0.01
Plasma fibrinogen (mg/dl)	353 ± 28	312 ± 33	295 ± 15

Data are means ± SE. *P < 0.01 vs. control subjects; †P < 0.001 vs. control subjects and CAD patients.

Susceptibility of LDL to oxidation in vitro. A blood sample for LDL preparation was collected in plastic tubes containing an anticoagulant/lipoprotein preservative solution comprised of 2.8 mmol/l EDTA, 62 µmol/l chloramphenicol, gentamicin sulfate (50 µg/ml, final concentration), and 10 mmol/l ε-amino-η-caproic acid as reported previously (23).

LDL was isolated using a rapid single-spin density gradient ultracentrifugation procedure as described previously (23). Briefly, the density of plasma was increased to 1.21 g/ml by the addition of solid potassium bromide, and an 11-ml aliquot was layered under a nitrogen-saturated salt solution at d = 1.019 g/ml in a Beckman VTi 50 polyallomer tube (Beckman Instruments, Palo Alto, CA). The samples were centrifuged at 50,000 rpm at 7°C for 2 h and 40 min. The LDL band was aspirated and stored under negative pressure at 4°C until analyzed.

The susceptibility to oxidation of LDL isolated from each patient and his or her matched control subject was determined the next day as described previously (23). Briefly, the aqueous antioxidants used in the preparation of LDL were first removed by gel filtration. After chromatography, the in vitro oxidation procedure was initiated within 1 h of the removal of the antioxidants. LDL (100 µg cholesterol/ml) was diluted in phosphate-buffered saline, which had been purged previously with oxygen, and the oxidation reaction was initiated by the addition of 5 µmol/l $CuCl_2$ (final concentration). The absorbance at 234 nm was continuously monitored in a Beckman DU 640 spectrophotometer (Beckman Instruments), with the sample compartment heated to 25°C. The lag time, maximal rate of oxidation, and extent of oxidation were calculated for each LDL sample assayed. The lag time was defined as the interval between initiation (time 0) and the intercept of the tangent of the slope of the absorbance curve during the propagation phase, with the time scale axis expressed in minutes. The maximal rate of oxidation was calculated from the slope of the absorbance curve during the propagation phase and was expressed as absorbance units per minute. The extent of oxidation was measured as the difference between the initial and the maximal absorbance at 234 nm.

Characterization of LDL. To determine the lipid composition of LDL isolated as described above, each LDL sample was extracted with chloroform: methanol (2:1, vol/vol), and the free and total cholesterol, triglyceride, phospholipid phosphorus, and protein concentrations were determined as described previously (24). The composition of fatty acids in LDL triglyceride, CE, and phospholipid was determined by gas chromatography as described previously (23). The concentrations of α-tocopherol, γ-tocopherol, α-carotene, β-carotene, and lycopene in LDL were determined using high-performance liquid chromatography as described previously (23). LDL particle size was determined using nondenaturing gradient gel electrophoresis of commercially available 3–13% gradient gels (Gradipore; Pyrmont, New South Wales, Australia) as described previously (23). The extent of glycation (25) and the concentration of thiobarbituric acid reactive substances (26) were also determined for each LDL sample.

Cell culture. Peripheral blood human monocytes were obtained from healthy normolipidemic volunteers as described previously (15). The purity of isolated monocytes obtained by this method ranged between 70 and 85%, and the contaminating cells were, in their totality, lymphocytes.

For experiments designed to measure cholesterol mass, monocytes were plated on 24-well tissue culture trays (Costar, Cambridge, MA) at a cell concentration of 1×10^6/ml in Iscove's modified Dulbecco's medium supplemented with 30% human serum and incubated for 6 days. The medium was changed every 3 days and, given the lack of adherence of the lymphocytes, 95% or more of the cells remaining attached to plastic at the end of the maturation period were esterase positive. To conduct an experiment, medium containing whole human serum was removed, the cells were washed, and the cells were incubated in medium with the same composition but without the addition of whole human serum. Our media and reagents were screened for endotoxin contamination using the E-toxate kit (Sigma, St. Louis, MO) and found to contain <0.015 endotoxin units/ml.

Measurements of total, free, and esterified cholesterol in macrophages. Macrophages (1×10^6 cells) were incubated, in triplicate, for 18 h with RBC-bound IC (20×10^6 cells). PEG-IC from each patient and his or her respective control were incubated concomitantly during the same experiment. In vitro prepared soluble and insoluble LDL-IC, made as previously described (8), were used as a positive control. After incubation, the medium was removed, the cells were extensively washed with phosphate-buffered saline, and the lipids of the cell monolayer were extracted with hexane/isopropanol (3:2) (vol/vol) as previously described (27). β-Stigmasterol was used as an internal standard. The lipid extracts of triplicate wells were pooled. Free and total cholesterol were assayed using gas chromatography as described previously (20). Cholesteryl ester levels were obtained by subtracting free cholesterol from total cholesterol levels. Cholesteryl ester accumulation by macrophages was expressed as micrograms of CE per milligram cell protein. After the lipid extraction, the cell pellet was solubilized with 0.2 mol/l NaOH containing 1% SDS, and the protein content was determined (19).

Other methods. Plasma glucose was assayed using the glucose oxidase method as adapted for use in the Beckman glucose analyzer, and plasma HDL cholesterol concentration was determined after precipitation of VLDL and LDL with sodium phosphotungstate:magnesium chloride as described previously (28). HbA_{1c} was quantitated after the separation of RBC hemolysates on columns packed with a weakly acidic cation exchange resin (Hemoglobin A_{1c} Micro Column Test; Bio-Rad, Hercules, CA). Total cholesterol and triglyceride was measured in whole plasma and lipoprotein fractions using enzymatic colorimetric assays (Sigma). Plasma fibrinogen levels were determined using the ACL-3000 automated analyzer (Instrumentation, Lexington, MA). Creatinine levels in urine and serum, and serum uric acid were determined using an EPX analyzer (Abbott, Chicago). Urinary albumin concentration (N/T Protein; Behring, Sommerville, NJ) was determined using nephelometry. Butylated-hydroxytoluene (40 µmol/l, final concentration) was added to aliquots of LDL, which were then frozen at –70°C for subsequent analysis of the lipid soluble antioxidants as described below.

Statistical analysis. Results are reported as means ± SE. Comparisons between diabetic patients, patients with CAD, and control subjects were analyzed statistically using 1-way analysis of variance with post hoc analyses, conducted using Dunnet's method for comparisons relative to the control group, or with Tukey's multiple comparisons method for all pairwise comparisons. For data not normally distributed, comparisons were investigated using Kruskal-Wallis 1-way analysis of variance on ranks. The analysis of paired data was conducted using the paired t test when appropriate. Linear regression analysis was conducted to analyze the relationship between different parameters and cholesterol ester accumulation in human macrophages. Statistical significance was assumed for P values <0.05. All calculations were performed with the SigmaStat statistical package, version 2.0 (SPSS, Chicago).

RESULTS

Anti-modified LDL antibodies. Antibodies to oxidized and glycated LDL were determined using a competitive enzymoimmunoassay as described in RESEARCH DESIGN AND METHODS. The levels of anti–ox-LDL and anti–gly-LDL antibodies did not differ significantly in the diabetic patient, CAD patient, and control groups (Fig. 1). It should be noted, however, that the levels of anti–ox-LDL antibodies were lower in the diabetic patient and CAD patient groups compared with the control group (483 ± 79, 379 ± 66, and 412 ± 63 ngEq/ml in the control, diabetic patient, and CAD patient groups, respectively). It should also be noted that general reactivity of anti–gly-LDL antibodies was very low. The mean ± SE of the OD values corresponding to the binding of specific anti–gly-LDL in

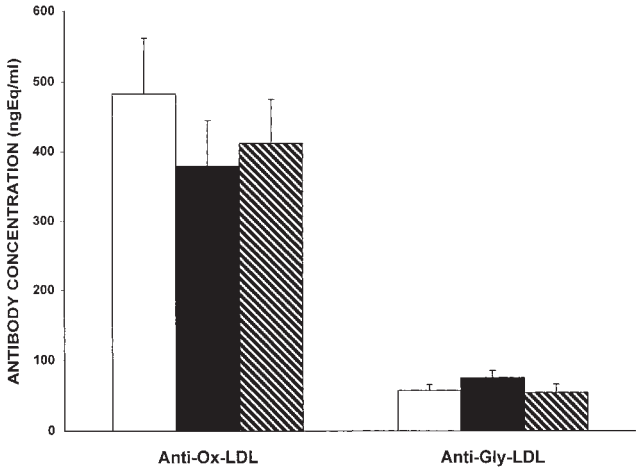

FIG. 1. Concentration of anti–ox-LDL and anti–gly-LDL antibodies in the serum of control subjects (□), type 2 diabetic patients with macrovascular complications (■), and nondiabetic patients with CAD (▨). The levels are expressed as nanogram equivalents per milliliter. Data shown are the mean ± SE for each group. Differences in values among groups were not statistically significant.

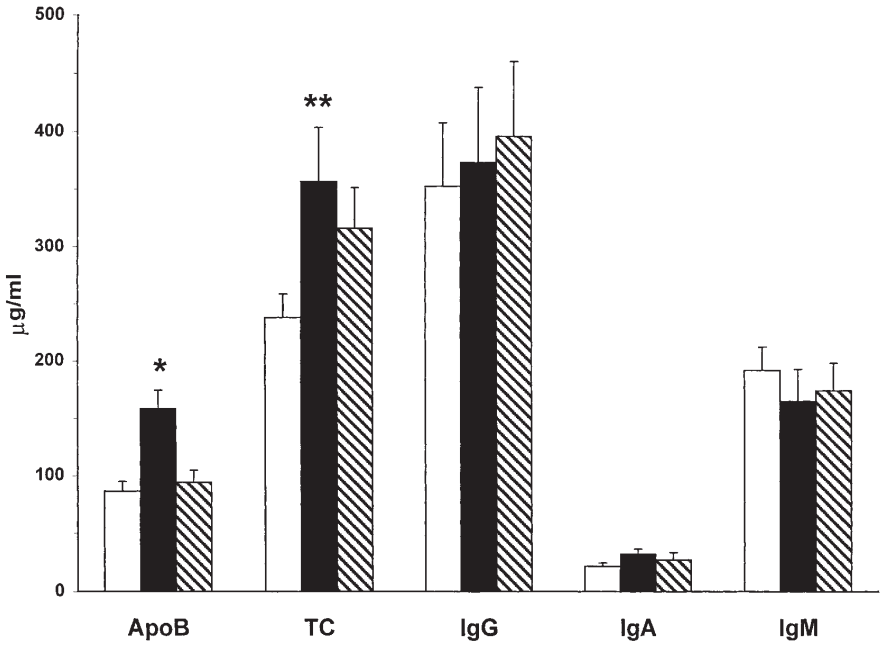

FIG. 2. Composition (apolipoprotein B, total cholesterol [TC], IgG, IgA, and IgM) of PEG-IC isolated from control subjects (□), type 2 diabetic patients with macrovascular complications (■), and nondiabetic patients with CAD (▨). The several components of PEG-IC were measured using the methodology described in RESEARCH DESIGN AND METHODS. The levels are expressed as micrograms per milliliter of resuspended PEG precipitate. Data shown are the mean ± SE for each group. *P < 0.001 vs. control and CAD; **P < 0.01 vs. control.

the combined group of patients and control subjects was 0.01 ± 0.001, whereas the OD values for specific anti–ox-LDL reactivity in the same combined group was 0.144 ± 0.013. The low degree of reactivity with gly-LDL was consistently observed in tests carried out using widely different concentrations of gly-LDL to coat the enzyme immunoassay plates.

Characterization of circulating IC. The composition of the PEG precipitates obtained from the sera of control subjects and diabetic and CAD patients is shown in Fig. 2. The precipitates obtained from subjects in the 3 groups contained variable but not statistically different amounts of IgG, IgM, and IgA. In contrast, there was a statistically significant increase in the content of cholesterol (P < 0.03) and apoB (P < 0.01) in the PEG-IC isolated from diabetic patients compared with those isolated from control subjects. There was no significant difference in the content of cholesterol in the PEG-IC isolated from diabetic patients compared with patients with CAD (357 ± 47 vs. 316 ± 35 ngEq/ml) or in CAD patients compared with control subjects (316 ± 35 vs. 238 ± 21 ngEq/ml). The content of apoB in the PEG-IC isolated from diabetic patients was also significantly higher than that in the PEG-IC isolated from patients with CAD (158 ± 16 vs. 94 ± 11 μg/ml, P < 0.01). The content of apoB did not differ significantly in the PEG-IC isolated from the patients with CAD compared with control subjects (94 ± 11 vs. 86 ± 8 μg/ml).

Evaluation of the atherogenic potential of isolated IC. The atherogenic potential of PEG-IC was determined by measuring the ability of PEG-IC to induce intracellular CE accumulation in human monocyte-derived macrophages. The assay was carried out using solubilized PEG-IC adsorbed onto O Rh^+ RBCs and incubated with human monocyte-derived macrophages as described in RESEARCH DESIGN AND METHODS. The extent of CE accumulation in human macrophages incubated with RBC-IC isolated from control subjects, diabetic patients, or patients with CAD is shown in Fig. 3. The intracellular CE concentration was significantly higher in macrophages incubated with RBC-adsorbed PEG-IC isolated from diabetic patients compared with that isolated from control subjects (29.9 ± 6.4 vs. 13.3 ± 1.2 μg CE/mg cell protein, P < 0.03) or from patients with CAD (16.0 ± 2.7 μg CE/mg cell protein, P < 0.04). There was no significant difference in the intracellular CE concentration in cells incubated with RBC-adsorbed PEG-IC isolated from the patients with CAD compared with control subjects.

To determine if differences in the composition of the PEG-IC among the 3 groups contributed to the observed differences in CE accumulation, we analyzed the correlations between the concentrations of constituents in the PEG-IC and the extent of CE accumulation in macrophages. No statistically significant correlations were found between intracellular CE concentration and the contents of IgG, IgA, or IgM in the PEG-IC. In contrast, the intracellular CE concentration was

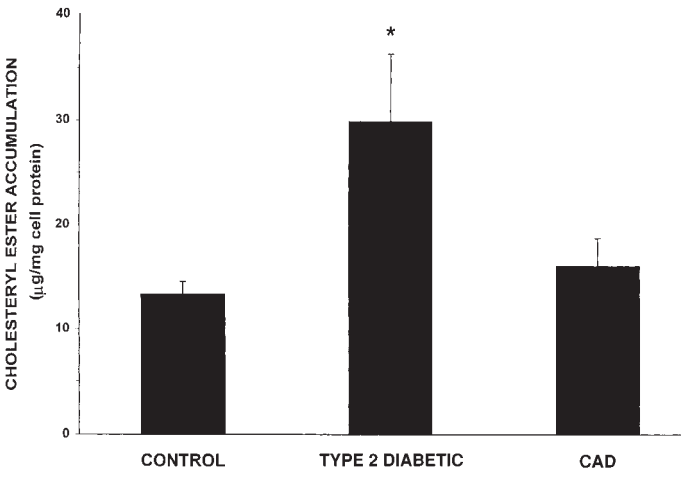

FIG. 3. CE accumulation in human macrophages incubated with RBC-IC isolated from control subjects, type 2 diabetic patients with macrovascular complications, and nondiabetic patients with CAD. RBCs (1×10^8) were incubated with 1.5 ml PEG-IC obtained from type 2 diabetic patients, CAD patients, and their respective control subjects, and washed and incubated with human monocyte–derived macrophages (1×10^6) for 18 h. Data shown are the mean ± SE of macrophage CE accumulation after incubation of the cells with RBC-IC from each of the 3 groups. *P < 0.05 vs. control and CAD.

TABLE 2
Parameters of copper-induced in vitro oxidation of LDL isolated from type 2 diabetic and CAD patients, and control subjects

Parameter	Type 2 diabetic patients	CAD patients	Control subjects
Lag time (min)	84.5 ± 8.7*	105.5 ± 10.1	98.0 ± 8.2
Rate (AU/min)	0.021 ± 0.001	0.024 ± 0.001	0.021 ± 0.001
Extent of oxidation (AU)	1.16 ± 0.09	1.22 ± 0.06	1.25 ± 0.09

Data are means ± SE. *P < 0.02 vs. control subjects. AU, absorbance units.

strongly correlated with the content of apoB (r = 0.68, P < 0.001) and cholesterol (r = 0.61, P < 0.001) in PEG-IC (Fig. 4).

Parameters of LDL composition and susceptibility to oxidation in vitro. Our next goal was to determine why patients with type 2 diabetes and macrovascular complications have an increased amount of LDL-IC. It is well known that glycoxidation is the major modification of LDL in diabetic patients; therefore, we compared the susceptibility of LDL obtained from subjects in each group to in vitro copper catalyzed oxidation. The lag time to oxidation in diabetic patients was significantly shorter than that in control subjects (P < 0.02). There were no significant differences in the rate of oxidation or in the extent of oxidation in LDL isolated from subjects in the 3 groups (Table 2). In addition, the mean initial and maximal absorbances at 234 nm also did not differ significantly among the 3 groups.

To determine if differences in the composition of LDL isolated from the diabetic patients made them more susceptible to oxidation and potentially more immunogenic, thus potentially facilitating the formation of LDL-IC in this group, we measured several parameters of in vivo modification of LDL isolated from the 3 groups. Because small dense LDL is more susceptible to oxidation and the presence of small-sized LDL is positively associated with risk of atherosclerosis, we determined the size of LDL isolated from the 3 groups. LDL isolated from diabetic patients was significantly smaller than LDL isolated from control subjects (P < 0.05) (Table 3). It also was smaller than LDL isolated from patients with CAD, but the difference did not reach statistical significance because of a smaller number of observations. In the presence of the high glucose concentrations commonly found in the serum of diabetic patients, LDL may become modified by glycation, and thus, may become more immunogenic. As expected, the extent of in vivo glycation of LDL isolated from the diabetic patients was greater than that of LDL isolated from control subjects, but the difference was not statistically significant. We did not find a significant difference in the extent of in vivo oxidation of LDL as demonstrated by similar levels of thiobarbituric acid reactive substances in LDL isolated from the 3 groups. Differences in the levels of the endogenous

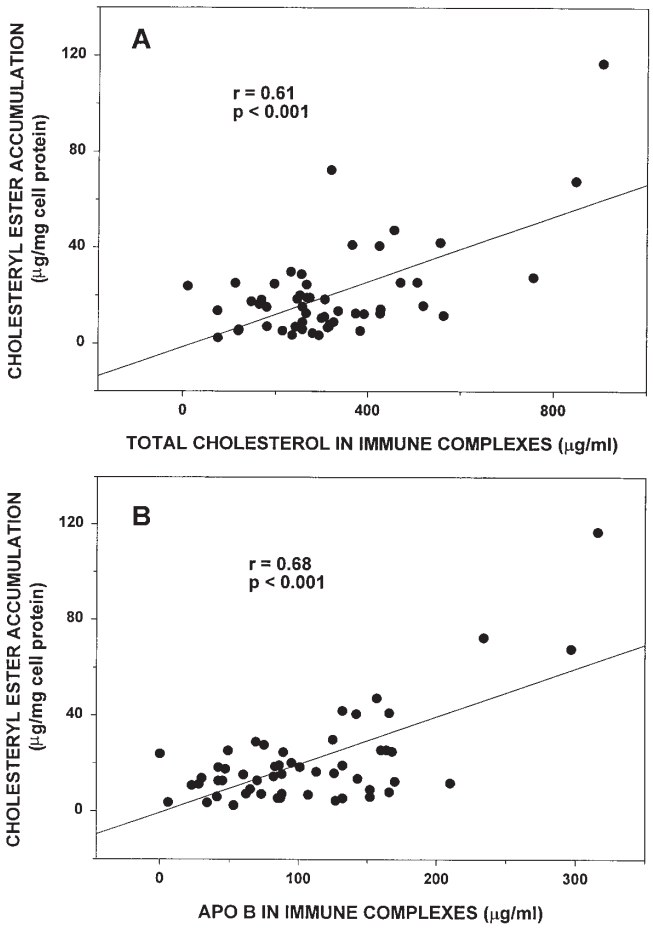

FIG. 4. Correlations between CE accumulation in human macrophages incubated with RBC-IC and the content of cholesterol (*A*) and apoB (*B*) in the PEG-IC. The *r* values indicated in the figure were calculated by linear regression analysis.

TABLE 3
Characteristics of LDL isolated from type 2 diabetic patients and CAD patients and control subjects

Parameter	Type 2 diabetic patients	CAD patients	Control subjects
Diameter (nm)	23.2 ± 0.2*	24.0 ± 0.2	23.9 ± 0.1
Glycation (cpm/nmol TNBSRS)	83 ± 4	ND	71 ± 5
Thiobarbituric acid reactive substance (pmol/mg LDL)	1,039 ± 164	725 ± 159	807 ± 132
α-Tocopherol (nmol/mg)	13.1 ± 1.3*	19.3 ± 1.5	20.7 ± 2.2
γ-Tocopherol (nmol/mg)	1.9 ± 0.5	1.7 ± 0.3	2.0 ± 0.3
Lycopene (nmol/mg)	0.16 ± 0.03	0.18 ± 0.03	0.23 ± 0.04
α-Carotene (nmol/mg)	0.03 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
β-Carotene (nmol/mg)	0.09 ± 0.05	0.08 ± 0.01	0.08 ± 0.01

Date are means ± SE. ND, not determined. *P < 0.05 vs. control subjects. TNBSRS, trinitrobenzenesulfonic acid reacting substance.

antioxidants in LDL may contribute to the susceptibility to oxidation of the particle. Therefore, we determined the antioxidant composition of LDL from each group (Table 3). Indeed, the concentration of α-tocopherol was significantly lower in LDL isolated from diabetic patients compared with that in LDL from control subjects or patients with CAD. There were no significant differences in the concentrations of γ-tocopherol, α-carotene, β-carotene, and lycopene in LDL isolated from the 3 groups.

During oxidation, polyunsaturated fatty acids are modified and form conjugated dienes. These reactive components will ultimately form lipid products, which will covalently bind to and modify LDL protein. We analyzed the CE, triglyceride, and phospholipid classes of LDL from each subject to determine if the fatty acid composition of the lipid classes differed among the 3 groups and thus might have contributed to the altered immunogenicity of LDL in diabetic patients. We found significant differences in the fatty acid composition of phospholipids and CEs (Table 4) of LDL isolated from the 3 groups. The content of monounsaturated fatty acids in phospholipids in LDL isolated from diabetic patients was significantly higher ($P < 0.05$) than that in LDL isolated from patients with CAD and control subjects. Whereas the total concentrations of polyunsaturated fatty acids did not differ significantly in the LDL isolated from the 3 groups, there was a significant ($P < 0.05$) increase in the n-3 polyunsaturated fatty acid content of phospholipids of LDL isolated from diabetic patients compared with patients with CAD and control subjects. The concentration of n-3 polyunsaturated fatty acids of CEs in LDL isolated from the diabetic patients was also significantly higher ($P < 0.05$) than that found in LDL isolated from CAD patients, whereas the concentration of n-6 polyunsaturated fatty acids was significantly lower. The concentration of n-3 polyunsaturated fatty acids in CEs of LDL isolated from CAD patients was significantly lower ($P < 0.05$) than that found in CEs of LDL from control subjects and patients with diabetes. There were no significant differences in the fatty acid composition of triglycerides in LDL isolated from the 3 groups.

DISCUSSION

Although it is generally accepted that the immune system actively contributes to both the onset and progression of atherosclerosis, the role of the various cells, antibodies, lymphokines, and other circulating proteins in the process leading to atheroma formation is poorly understood (29,30). Data has accumulated in recent years suggesting that an autoimmune response triggered by the modification of LDL, leading to the production of antibodies to modified LDL and to immune complex formation, may be a key event in the pathogenesis of atherosclerosis (31). It also has been postulated that the role of immune mechanisms in the pathogenesis of macrovascular complications in diabetes may be even more important than in the development of cardiovascular disease in the general population (5). Considering that protein glycation and glucose auto-oxidation, common events in diabetic patients, are known to generate free radicals and thus may catalyze lipid peroxidation (2,13,14), the possible involvement of autoantibodies to modified LDL and LDL-IC in the development of macrovascular complications in diabetic subjects appeared worthy of investigation.

The presence of antibodies against oxidized and glycated LDL in the serum of patients with diabetes has been reported by several investigators (32–36), including us (15). However, there is considerable disagreement between different groups about the nature of the abnormalities in modified LDL antibody levels in diabetic patients. Whereas Bellomo et al. (32) reported higher levels of autoantibodies against glycated and oxidized LDL in type 2 diabetes patients than in control subjects and Festa et al. (34) found a higher level of autoantibodies to oxidized LDL in type 1 diabetes patients, other investigators (33,36), including ourselves (15), have found comparable levels of these antibodies in type 1 as well as type 2 diabetic patients. In the present study, we also did not find any significant difference in the levels of free circulating anti–ox-LDL and anti–gly-LDL antibodies in the diabetic patient group as compared with the CAD patient and control groups (Fig. 1). Although not statistically significant, the lev-

TABLE 4
Fatty acid composition of phospholipid, CE, and triglyceride in LDL isolated from type 2 diabetic patients and CAD patients and control subjects

	Phospholipid			CE			Triglyceride		
Fatty acid	Type 2 diabetic patients	CAD patients	Control subjects	Type 2 diabetic patients	CAD patients	Control subjects	Type 2 diabetic patients	CAD patients	Control subjects
14:0	3.7 ± 1.2	0.4 ± 0.1	1.1 ± 0.5	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	1.5 ± 0.3	2.0 ± 0.6	6.5 ± 0.6
16:0	19.4 ± 2.3	23.2 ± 2.3	23.4 ± 1.6	12.0 ± 0.6	12.8 ± 0.4	11.5 ± 0.5	20.8 ± 1.6	23.5 ± 1.6	21.1 ± 1.9
16:1(n-7)	6.2 ± 2.3	0.5 ± 0.1	2.7 ± 1.2	1.5 ± 0.2	2.3 ± 0.2	2.6 ± 0.3	1.4 ± 0.2	1.9 ± 0.2	2.5 ± 0.6
18:0	15.5 ± 0.7	22.3 ± 1.6	18.7 ± 0.9	1.6 ± 0.2	1.5 ± 0.1	1.5 ± 0.1	8.3 ± 0.7	8.4 ± 0.8	8.6 ± 0.6
18:1(n-9)	14.8 ± 1.9	11.6 ± 0.7	12.7 ± 0.4	17.5 ± 0.7	19.7 ± 0.8	19.6 ± 0.6	39.1 ± 2.3	41.4 ± 1.9	37.7 ± 1.5
18:2(n-6)	17.5 ± 1.4	18.2 ± 0.9	19.2 ± 0.7	44.1 ± 3.4	47.5 ± 1.6	47.3 ± 1.5	14.6 ± 1.0	14.6 ± 0.7	13.1 ± 0.9
20:4(n-6)	7.8 ± 0.9	7.7 ± 0.8	7.5 ± 0.6	6.7 ± 1.2	6.7 ± 0.6	5.5 ± 0.5	2.6 ± 0.4	1.8 ± 0.2	2.9 ± 0.7
Saturated	37.4 ± 1.9	45.6 ± 2.3	42.6 ± 1.6	14.0 ± 0.8	14.8 ± 0.5	13.5 ± 0.5	29.7 ± 1.8	32.3 ± 2.1	33.1 ± 1.8
Monounsaturated	22.2 ± 2.5*†	14.0 ± 0.8	16.1 ± 1.0	20.3 ± 0.9	22.8 ± 0.9	23.0 ± 0.8	42.4 ± 2.3	44.4 ± 2.1	42.2 ± 1.4
Polyunsaturated	31.6 ± 3.3	28.1 ± 1.6	29.4 ± 1.4	51.9 ± 3.5	55.4 ± 1.5	55.0 ± 1.7	16.9 ± 1.2	16.4 ± 0.8	16.7 ± 0.9
Polyunsaturated (n-3)	7.0 ± 2.0*†	2.2 ± 0.9	3.3 ± 0.7	7.3 ± 1.3†	1.0 ± 0.1‡	3.7 ± 0.6	0.8 ± 0.1	0.8 ± 0.1	2.0 ± 0.4
Polyunsaturated (n-6)	25.2 ± 2.0	25.9 ± 1.5	25.8 ± 1.1	44.3 ± 3.3†	54.2 ± 1.6	50.9 ± 1.6	16.3 ± 1.2	16.1 ± 0.8	14.9 ± 0.9

Data are means ± SE. Saturated = 14:0 + 16:0 + 18:0; monounsaturated = 16:1(n-9) + 16:1(n-7) + 18:1(n-9) + 18:1(n-7); polyunsaturated = 18:2(n-9) + 18:2(n-6) + 20:2(n-6) + 20:4(n-6) + 20:5(n-3) + 22:5(n-3) + 22:6(n-3). *$P < 0.05$, type 2 diabetic patients vs. control subjects; †$P < 0.05$, type 2 diabetic patients vs. CAD patients; ‡$P < 0.05$, CAD patients vs. control subjects.

els of anti–ox-LDL antibodies in both diabetic and nondiabetic groups with coronary atherosclerosis were lower than those in the control group.

The level of anti–gly-LDL antibodies in the diabetic patient group with a long duration of diabetes and macrovascular complications was not significantly higher than in subjects without diabetes. In addition, the general reactivity of these antibodies was extremely low compared with reactivity of anti–ox-LDL antibodies. These data support our previous observation in type 1 diabetic patients (15) and allow us to conclude that gly-LDL is poorly immunogenic and that anti–gly-LDL antibodies probably play a minimal role in atherogenesis.

We have previously proposed that the pathogenic role of anti-LDL antibodies derives from the formation of atherogenic LDL-IC (5). We have demonstrated the presence of LDL-IC in the sera of type 1 diabetic patients without complications (15). Recently, Festa et al. (34) reported the presence of LDL-IC in the sera of type 1 diabetic patients with microvascular complications. We have also shown that these IC contained anti–ox-LDL antibody and that their atherogenic potential, i.e., their ability to induce CE accumulation in macrophages, was directly related to their content of apoB and cholesterol. Furthermore, we have also shown that the apoB content of the IC could predict the development of macrovascular disease in type 1 diabetes (37). A paradoxical relationship between the levels of free anti–ox-LDL antibody and LDL-IC in the sera of diabetic patients has been underlined by Festa et al. (34) and by our group (16,37). Festa et al. reported that in type 1 diabetic patients, LDL-IC were detected mostly in anti–ox-LDL antibody–negative patients as compared with anti–ox-LDL antibody–positive patients. We have recently shown (37) that patients with type 1 diabetes who developed CAD during an 8-year follow-up period had significantly higher levels of LDL-IC than patients who did not develop CAD. Most importantly, the concentration of these IC inversely correlated with the level of free anti–ox-LDL antibodies in serum. This finding suggests that the formation of LDL-IC interferes with the assay of free anti–ox-LDL antibodies, and the paradoxical decrease of anti–ox-LDL antibodies in patients with CAD or microvascular complications is a consequence of such interference.

LDL-IC is an important pathogenic factor for the development of atherosclerosis in diabetic patients. This is evidenced by our observation that LDL-IC isolated from patients with type 2 diabetes and macrovascular complications are more atherogenic in vitro than LDL-IC isolated from healthy volunteers or even from nondiabetic patients with CAD (Fig. 1). The explanation for this difference appears to reside in differences in the composition of the PEG precipitates. PEG-IC isolated from diabetic patients contained significantly higher amounts of cholesterol and apoB than those isolated from control subjects or patients with CAD. A similar observation has been previously made in a group of type 1 diabetes patients (15), and the sum of these studies strongly suggests that the ability of LDL-IC to induce more CE accumulation in macrophages is mostly dependent on their cholesterol and apoB contents.

It should be noted that the study population of type 2 diabetic patients exhibited significantly elevated levels of plasma triglycerides (Table 1). Hypertriglyceridemia is frequently associated with type 2 diabetes. Most importantly, it is also associated with increased cardiovascular risk. To determine the relative risk of hypertriglyceridemia on the parameters measured in this study, we also examined the data with the hypertriglyceridemic patients excluded. The 6 patients with hypertriglyceridemia (plasma triglyceride level >250 mg/dl) did not have significantly different levels of serum antibodies to oxidized or glycated LDL. The concentrations of apoB and cholesterol in PEG-IC remained significantly elevated in the population of diabetic patients when the hypertriglyceridemic patients were excluded. Likewise, CE accumulation in human macrophages incubated with PEG-IC remained significantly elevated in the population when the diabetic hypertriglyceridemic patients were excluded.

Having established the correlation between LDL-IC and the ability to cause CE accumulation in human macrophages, we turned our attention to the investigation of the factor(s) that may determine the presence of higher levels of circulating LDL-IC in the diabetic patients. One logical assumption would be that LDL in diabetic patients is more prone to undergo chemical modification as a consequence of glycation, and thus the formation of antibodies to modified LDL and LDL-IC could be facilitated. To test this hypothesis, we compared different parameters of LDL composition and the extent of LDL modification in the 3 groups of subjects.

LDL glycation does not appear to have contributed to the increased susceptibility to oxidation of LDL from diabetic patients because the extent of glycation of this LDL was not significantly greater than that for LDL isolated from matched control subjects (Table 3).

It is well established that small dense LDL is positively associated with increased risk of atherosclerosis, and its presence is common in diabetic patients, especially in patients with hypertriglyceridemia (2,38). Small dense LDL is more susceptible to oxidation and is associated with higher levels of malondialdehyde-LDL autoantibodies than large LDL (39). A decrease in the endogenous antioxidant concentration in LDL is also associated with increased susceptibility of the particle to oxidation (40). In our study, we found that LDL isolated from the diabetic patients was significantly smaller than LDL isolated from control subjects and contained a decreased amount of α-tocopherol (Table 3). These findings were compatible with the finding that LDL from diabetic patients was significantly more susceptible to in vitro copper-induced oxidation than LDL isolated from matched control subjects (Table 2). LDL modified by copper-induced oxidation closely mimics LDL modified by cell-mediated oxidation (41). Furthermore, the addition of copper ions to stimulate peroxidation of LDL in vitro may be a valid model for events in the arterial wall because advanced human arteriosclerotic lesions contain iron and copper in forms capable of catalyzing lipid peroxidation (42). LDL from the diabetic patients, based on its increased susceptibility to oxidation in our in vitro copper-mediated oxidation assay, may more readily supply an antigenic stimulus for antibody formation in vivo and thus contributes to the elevated concentrations of cholesterol and apoB in IC found in these patients (Fig. 2).

Although there were significant differences in the susceptibility of LDL to oxidation, there were no significant differences in the rate of oxidation or in the extent of oxidation of LDL among the 3 groups (Table 2). Whereas numerous studies have investigated the factor(s) that influence the lag time to oxidation of LDL, few studies have investigated the mech-

anisms of the altered rate or of the extent of LDL oxidation. Previous studies have shown that dietary supplementation of oleic acid increases its content in LDL, and this correlates strongly with the extent of conjugated diene formation (extent of oxidation) (43,44). These studies were confirmed and extended to demonstrate that the ratio of oleic acid to linoleic acid content of LDL was inversely correlated with the rate and extent of oxidation (45,46). There were no significant differences in the contents of oleic acid or linoleic acid or the ratio of the masses of these two fatty acids in LDL triglyceride, CEs, or phospholipids among the 3 groups (Table 4). Additional studies have revealed a strong positive correlation between the ratio of cholesterol mass to protein mass in LDL and both the oxidation rate and the extent of oxidation (4). There was no significant difference in the chemical composition of LDL among the 3 groups (data not shown). In sum, our determinations of LDL composition support the observed lack of difference in the rate and extent of oxidation of LDL among the 3 groups. However, determination of the extent of oxidation of LDL is critical. LDL oxidation may be likened to a bomb with a burning fuse. The susceptibility to oxidation may be short or long depending on the length of the fuse. However, when LDL oxidizes and the bomb finally explodes, it is the size of the bomb (extent of oxidation) that will ultimately determine the damage that will ensue.

There were additional significant differences in the composition of the fatty acids of the different lipid classes of LDL in the 3 groups (Table 4). It is interesting to note that Griffin et al. (35) observed higher levels of antibodies to modified LDL in patients with increased levels of polyunsaturated fatty acids in LDL and that Kontush et al. (40) reported that the content of n-3 polyunsaturated fatty acid is the most important determinant of the oxidizability of LDL. Whereas we found no significant differences in the total concentrations of polyunsaturated fatty acids in lipids among the 3 groups, the concentration of n-3 polyunsaturated fatty acids in phospholipids and CEs of LDL isolated from diabetic patients was significantly higher than that measured in LDL isolated from patients with CAD or from control subjects. Thus, the increased concentration of n-3 polyunsaturated fatty acids in phospholipids and CEs of LDL isolated from the diabetic patients is consistent with the increased susceptibility to oxidation of LDL from these patients.

Altered fatty acid composition of lipids in LDL from the diabetic patients may also contribute to LDL antigenicity in another manner. The first step in the oxidative modification of LDL is lipid peroxidation of polyunsaturated fatty acids, followed by oxidative chain reactions and cleavage of fatty acids. These reactions lead to the formation of reactive fragments, such as malondialdehyde and 4-hydroxy-nonenal. These reactive fragments will become linked with lysine residues and form epitopes that differentiate oxidized proteins from their native counterparts and render them immunogenic. In addition, the structure of fatty acid fragments formed during the oxidative metabolism of n-3 compared with n-6 fatty acids will differ. We postulate that the increased n-3 fatty acid content in LDL isolated from type 2 diabetic patients by facilitating the formation of propanyl-lysine adducts instead of the hexanoyl-lysine adducts derived from n-6 fatty acids enhances the immunogenicity of ox-LDL. The increased content of cholesterol and apoB in the LDL-IC isolated from type 2 diabetic patients is certainly consistent with an increased immunogenicity of ox-LDL in those patients. Alternatively, the propanyl-lysine adduct-modified LDL could elicit antibodies with higher affinity and thus facilitate the formation of IC. If this hypothesis proves true, the concentration and chemical nature of polyunsaturated fatty acids in LDL may play a critical role in production of antibodies to oxidized LDL and consequently in the formation of LDL-IC.

The factor(s) that contributed to the altered antioxidant content and fatty acid composition in LDL from diabetic patients remains to be determined. The effects of dietary intake per se on the content of α-tocopherol in LDL and the effect of the LDL α-tocopherol content on LDL oxidation have been repeatedly confirmed and can be considered as unequivocal (47). However, increased oxidative stress in diabetes also may serve to reduce endogenous antioxidant levels in LDL and thus contribute to increased susceptibility to oxidation (48). Several studies have suggested possible links between the altered glucose metabolism in diabetes and changes in fatty acid composition and metabolism in skeletal muscle (49–51). The link between altered tissue fatty acid composition and lipoprotein lipid fatty acid composition remains to be demonstrated.

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