Sensitive detection of oxidatively modified low density lipoprotein using a monoclonal antibody

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Abstract We have established a new method capable of measuring the very low concentrations of oxidized low density lipoprotein (OxLDL). In our previous study, we obtained a novel murine monoclonal antibody against oxidized lipoproteins (Itabe, H. et al. 1994. *J. Biol. Chem.* **269:** 15274–15279). The epitope of this antibody resides in oxidized products of phosphatidylcholine that can form complexes with polypeptides, including apolipoprotein B. When the monoclonal antibody was precoated onto microtiter wells prior to carrying out a sandwich ELISA using an anti-human apolipoprotein B antibody, it was possible to detect 0.5 ng protein of copper-induced OxLDL. The detection of OxLDL was dependent on the presence of monoclonal antibody and was blocked by oxidized phosphatidylcholine (OxPC). Under the same sandwich ELISA condition, native LDL showed a dose-dependent increase of absorbance that was inhibited by complex of OxPC with BSA. These results suggest the possible occurrence of oxidative modification of human plasma LDL which is recognized by the antibody against OxPC. The level of LDL oxidation of normal human subjects was found to be 0.52 ± 0.35 units per 5 μg protein of LDL, where one unit was defined as the reactivity corresponding to 1 ng of copper-induced OxLDL by this assay. Furthermore, we found that the LDL oxidation level in patients who had been receiving hemodialysis treatment was increased more than eightfold over that of normal subjects. We suggest that LDL in human plasma is oxidatively modified under certain conditions and this method for measurement of OxLDL could be used to study the relationship between in vivo oxidation reaction and various pathological conditions.—**Itabe, H., H. Yamamoto, T. Imanaka, K. Shimamura, H. Uchiyama, J. Kimura, T. Sanaka, Y. Hata, and T. Takano.** Sensitive detection of oxidatively modified low density lipoprotein using a monoclonal antibody. *J. Lipid Res.* 1996. **37:** 45–53.

Supplementary key words oxidized LDL • LDL oxidation levels in human plasma • monoclonal antibody • sandwich ELISA • renal dysfunction • hemodialysis

The involvement of lipid peroxidation in a number of pathophysiological conditions, such as atherosclerosis (1, 2), ischemia-induced cerebral injury (3, 4), chronic renal dysfunction (5, 6), and diabetes (7, 8) has been well documented. In particular, oxidatively modified low density lipoprotein (LDL) has been shown to be involved in the initiation and promotion of atherosclerosis (9–11). Formation of lipid-laden foam cells from macrophages has been demonstrated by incubating oxidized LDL (OxLDL) with macrophages in vitro (12, 13), while incubation with native LDL did not result in accumulation of lipid droplets. OxLDL also exhibited a number of cell biological activities including enhancement of interaction of leukocytes and endothelial cells (14, 15), inhibition of endothelial cell migration (16), and induction of endothelin secretion from endothelial cells and macrophages (17, 18).

OxLDL prepared with copper ion in vitro has been widely used as a model for modified LDL. Although its structure has not been fully characterized, LDL extracted from atherosclerotic lesions has been shown to share many of the characteristics of copper-induced OxLDL (19). Monitoring OxLDL levels in human plasma would be very useful for studying atherogenesis and LDL metabolism as well as for diagnostic purposes. Unfortunately, it has been very difficult to measure low levels of oxidatively modified LDL in the presence of native LDL. However, a monoclonal antibody obtained in our previous study (20), FOH1a/DLH3, showed good reactivity to OxLDL but did not react with chemically modified LDLs such as acetylated LDL or malondialde-

Abbreviations: LDL, low density lipoprotein; OxLDL, oxidized low density lipoprotein; PC, phosphatidylcholine; OxPC, oxidized phosphatidylcholine; apoB, apolipoprotein B; LPDP, lipoprotein-deficient plasma; BHT, butylhydroxytoluene; MDA, malondialdehyde; TBA, thiobarbituric acid; ALP, alkaline phosphatase; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; HSA, human serum albumin; PBS, phosphate-buffered saline; TBS, Tris-HCl-buffered saline.

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hyde (MDA)-treated LDL. In the present study we have used FOH1a/DLH3 to develop a new sensitive method for measuring oxidative modification of human plasma LDL. We found a higher level of oxidation in the plasma LDL fraction from patients treated with hemodialysis than in plasma LDL from normal subjects.

MATERIALS AND METHODS

Materials

Sheep anti-human apolipoprotein B (apoB) antibody (IgG fraction) was purchased from Binding Site Inc., Birmingham, UK. Alkaline phosphatase (ALP)-conjugated goat anti-sheep IgG antibody, which was preabsorbed with human, rat, bovine, and murine serum, was from Chemicon Co., Temecula, CA. Two types of BSA preparation (fraction V and fatty acid-free), HSA, bovine fibronectin, human fibrinogen, and 1-palmitoyl-2-linoleoyl phosphatidylcholine were purchased from Sigma Chemical Co., St. Louis, MO. Vitronectin and *p*-nitrophenylphosphate were purchased from Wako Pure Chemical Co., Osaka, Japan. Type I collagen was purchased from Koken Co., Tokyo, Japan.

Preparation of monoclonal antibody FOH1a/DLH3

The monoclonal antibody FOH1a/DLH3 (murine IgM) was established as described previously (20). The antibody used in this study was prepared from ascites of mice that had received the corresponding hybridoma intraperitoneally. The ascites was centrifuged at 2,000 rpm for 10 min to remove cells and debris, and immunoglobulins in the supernatant were precipitated by adding ammonium sulfate up to 50% saturation. The precipitate was redissolved in a small volume of 10 mM borate buffer, pH 8.5, containing 150 mM NaCl, and the IgM was partially purified on a Toyopearl HW-65® (Tosoh, Tokyo, Japan) gel filtration column chromatography. Nonimmune murine IgM was prepared from normal murine serum by the same procedure as for the monoclonal antibody.

Preparation of OxLDL standard

LDL was separated from human plasma by sodium bromide stepwise density gradient centrifugation (21). All sodium bromide stock solutions contained 150 mM sodium chloride and 0.25 mM EDTA. After centrifugation, the fractions with a density of 1.019–1.063 g/ml were pooled as LDL. This LDL fraction was dialyzed against PBS containing 0.25 mM EDTA. When OxLDL was prepared, an aliquot of the LDL fraction was passed through a 10DG® desalting column (Bio-Rad) to remove the EDTA, then the LDL (0.2 mg/ml, diluted in PBS) was incubated with 5 μM $CuSO_4$ at 37°C for 3 h. The reaction was stopped by adding EDTA to a final concentration of 0.25 mM. The OxLDL prepared under these conditions showed an increase in relative mobility (1.8 ± 0.2) on agarose gel electrophoresis and 56.7 ± 9.7 nmol of thiobarbituric acid (TBA) reactive substances per mg protein.

Measurement of OxLDL

Blood samples were obtained intravenously using EDTA as anti-coagulant from healthy volunteers and patients receiving hemodialysis at Kimitsu Hospital, Chiba, Japan. The patients' samples were taken after hemodialysis treatment. For measurement of oxidation of human plasma LDL, and LDL fraction was obtained by sequential centrifugation (22). PBS (250 μl) containing 0.25 mM EDTA was layered on top of 750 μl of plasma and the tubes were centrifuged at 100,000 rpm (550,000 *g*) for 7 min. The top 250 μl of the sample was discarded to remove chylomicrons. A further 250 μl of PBS containing 0.25 mM EDTA was layered onto the remaining 750 μl, and the samples were centrifuged at 100,000 rpm for 2.5 h to float up VLDL. The top 250 μl was discarded again and 150 μl of 50 % (w/v) potassium bromide added to the remainder to give a mixture with a density of 1.063 g/ml. After a final centrifugation at 100,000 rpm for 5 h, the top layer (200 μl) was recovered as the LDL fraction. The LDL fraction was dialyzed against PBS containing 0.25 mM EDTA before use. From 750 μl of plasma amount of LDL recovered was ranging 0.3 to 0.7 mg protein.

One hundred microliters of diluted monoclonal antibody FOH1a/DLH3 (3 μg/ml in PBS) was incubated in each microtiter well (Flexible assay plate®, Falcon 3912) at room temperature for 2 h. After the antibody solution was discarded, the wells were filled with TBS containing 1% BSA (fraction V) at room temperature for at least 2 h. After the blocking solution was discarded, samples (human LDL fractions, 5 μg protein/well) or diluted standard OxLDL (0.05–20 ng/well) were applied to the well, and incubated at 4°C over night. Note that the wells were not washed with a buffer containing detergent before the sample was added to the antibody in this assay condition. The wells were then washed three times with TBS containing 0.05% Tween 20. The wells were incubated for 2 h at room temperature with 100 μl of sheep anti-human apoB antibody solution (diluted 1:5000 in PBS). After washing the wells with TBS-Tween three times, 100 μl of ALP-conjugated goat anti-sheep IgG antibody (diluted 1:10,000 in TBS containing 2% skim milk) was added for another 2 h. The reactivity of ALP was finally measured by incubating with 100 μl of *p*-nitrophenylphosphate (1 mg/ml, dissolved in 1 M triethanolamine buffer, pH 9.8) at 37°C for appropriate intervals and the absorbance was measured on an ELISA

plate reader (Bio-Rad, model 2550). Each sample was run with a control well that was precoated with nonimmune murine IgM (0.3 μg/well) instead of the monoclonal antibody. The absorbance obtained with the samples was corrected by subtracting the corresponding absorbance of the control wells. In each ELISA plate, various concentrations of standard OxLDL were run simultaneously to determine a standard curve.

Preparation of oxidized PC

Oxidized PC (OxPC) was prepared as described previously (20). Briefly, 1-palmitoyl-2-linoleoyl PC was suspended in PBS (2 mM) after being dried under argon gas. Ascorbic acid and $FeSO_4$ were dissolved in distilled water just before use. The reaction mixture, containing 0.4 mM PC, 0.4 mM ascorbic acid, and 40 μM $FeSO_4$ in PBS, was incubated at 37°C for 3 h under air with mild agitation. The reaction was stopped by addition of BHT (final 0.2 mM).

Preparation of oxidized PC-BSA complex

Aqueous suspension of 1-palmitoyl-2-linoleoyl PC was oxidized by incubation with ascorbic acid and $FeSO_4$ as described above but in the presence of BSA (0.2 mg/ml). After the reaction was stopped by addition of BHT (final 0.2 mM), the reaction mixture was lyophilized. The residue redissolved in a small volume of water was filtered through a 0.45-μm cartridge before being subjected to gel permeation HPLC. The HPLC was performed with a column G-3000SWXL (Tosoh, Tokyo, Japan), eluted with phosphate-buffered saline at 0.5 ml/min. The peak corresponding to standard BSA was recovered and used as OxPC-BSA complex.

Other methods

Protein concentration was determined by the bicinchoninic acid (BCA) method (23). Cholesterol concentration was measured by the cholesterol oxidase method (24). TBA reactive substance was measured by the method of Buege and Aust (25). Agarose gel electrophoresis was performed by the method of Noble (26). The level of phosphorous from PC was determined by the method of Zhou and Arthur (27).

RESULTS

Determination of OxLDL by a sandwich ELISA method

When OxLDL was coated directly onto a plastic microtiter well it was possible to detect down to 0.1 μg protein of OxLDL with the monoclonal antibody FOH1a/DLH3. As only limited sample (approximately 1 to 2 μg/well) can be absorbed onto microtiter wells, it is difficult to detect the very low levels of OxLDL that are present in the plasma LDL fraction. However, the sandwich ELISA methodology offers increased sensitivity as the precoated antibody binds OxLDL specifically, even in the presence of other components.

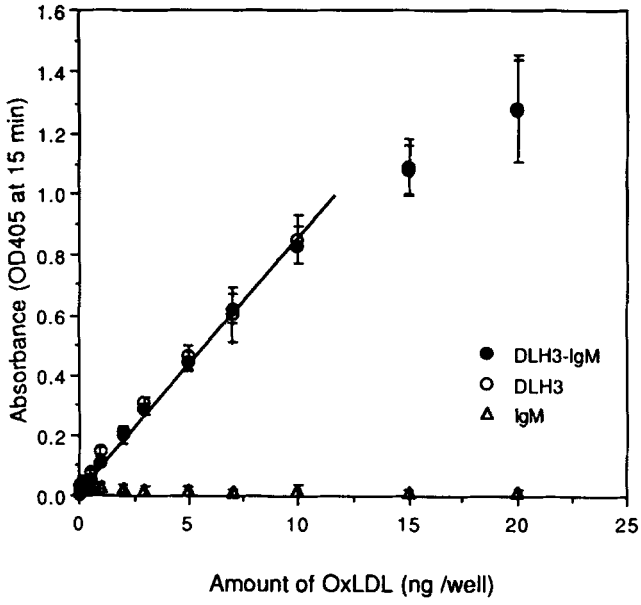

Fig. 1. Determination of OxLDL by a sandwich ELISA using the monoclonal antibody FOH1a/DLH3. Standard curve of the OxLDL determination showing the dose-dependent response, up to 10 ng, of OxLDL. OxLDL was prepared by incubating LDL with $CuSO_4$ at 37°C for 3 h. Microtiter wells were precoated with 0.3 μg of the monoclonal antibody FOH1a/DLH3 (open circle) or nonimmune murine IgM (open triangle). After the unoccupied surfaces of the microtiter wells were blocked with BSA, 100 μl of various concentrations of OxLDL was added to the microtiter wells. OxLDL particles in samples that were trapped by the precoated monoclonal antibody were determined by anti-human apoB antibody (sheep IgG) together with ALP-conjugated anti-sheep IgG antibody (donkey IgG). Values obtained with the nonimmune IgM were subtracted from the corresponding values of the monoclonal antibody FOH1a/DLH3 (closed circle). Values are mean ± standard deviation of three independent experiments.

Samples were added to the microtiter wells that had been precoated with the monoclonal antibody. After extensive washing, the remaining OxLDL was detected by an anti-human apoB polyclonal antibody together with an ALP-conjugated second antibody. The absorbance was proportional to the amount of OxLDL, up to 10 ng protein (**Fig. 1**). As little as 0.5 ng per well of OxLDL could be measured, a 100-fold increase in sensitivity over the direct ELISA method. When nonimmune murine IgM was precoated instead of the monoclonal antibody, almost no absorbance was observed. The high sensitivity was obtained because a single OxLDL particle attached to the precoated FOH1a/DLH3 can bind a large number of anti-apoB polyclonal antibody molecules.

The structures of the antigenic OxPC molecule(s) and other structures modified in OxLDL are as yet unclear.

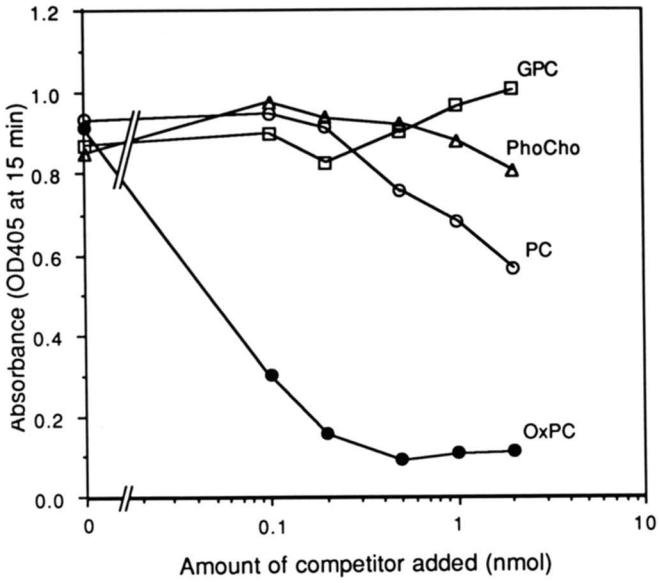

Fig. 2. Inhibition by OxPC of the sandwich ELISA for the determination of OxLDL. OxLDL (10 ng) mixed with various concentrations of PC analogs including 1-palmitoyl-2-linoleoyl PC (open circle; PC), oxidized 1-palmitoyl-2-linoleoyl PC (closed circle; OxPC), phosphocholine (open triangle; PhoCho) and glycerophosphocholine (open square; GPC) was added to the microtiter well precoated with the monoclonal antibody FOH1a/DLH3. The sandwich ELISA was performed as described in the legend of Fig. 1.

There is, therefore, no clearly defined standard for quantitative purposes. We decided to use copper-induced OxLDL prepared under a controlled condition as a standard material throughout this study. The extent of oxidative modification of LDL is dependent upon a number of factors, including the LDL/copper ratio (not on the copper concentration alone), remaining EDTA, temperature, and freshness of the copper solution. The OxLDL used in this study was prepared by incubating 0.2 mg/ml of LDL with 5 μM of copper sulfate at 37°C for 3 h. The reproducibility of the standard curve in the assay using different preparations of OxLDL was very good (Fig. 1). OxLDL standards were run with each ELISA plate.

To ensure the specificity of trapping of OxLDL by the monoclonal antibody, the effect of various PC analogs, including oxidized PC (OxPC) was investigated (**Fig. 2**). The epitope of the monoclonal antibody appeared to be OxPC product(s), and OxPC inhibited binding of the antibody to an OxPC-peptide complex used as a model antigen (20). Figure 2 shows that OxPC inhibited the binding of OxLDL to the monoclonal antibody, suggesting that the OxLDL in this sandwich ELISA was specifically bound to the monoclonal antibody.

In order to apply this method to the measurement of OxLDL in various samples, interference by other components of plasma and vessel wall was investigated. OxLDL (10 ng) was subjected to the ELISA assay in the presence of various proteins or complex sugars, including bovine and human albumins, globulin and fibrinogen (**Fig. 3**). Even at concentrations up to 1000-fold greater than that of OxLDL in the well, these materials only produced small changes in the ELISA reactivity. This suggests that the small amount of contaminating

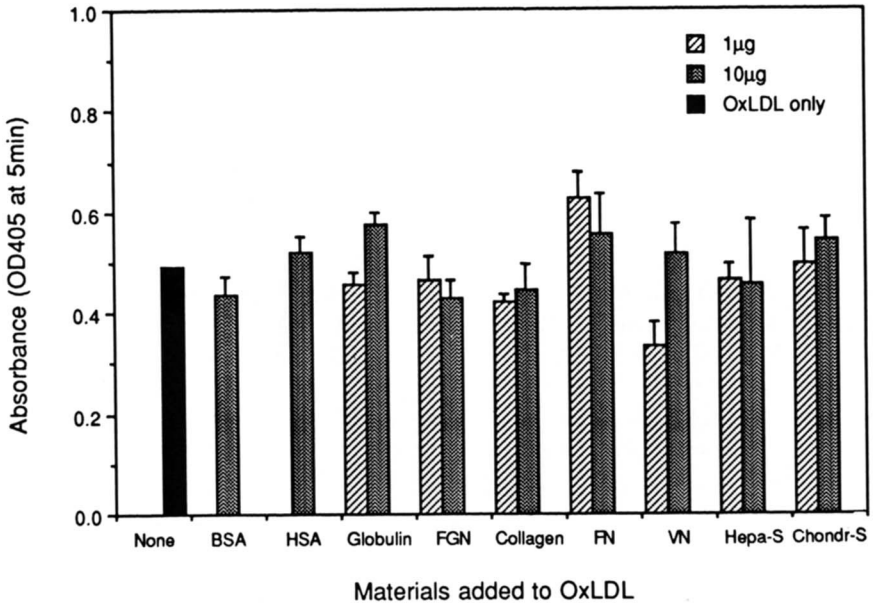

Fig. 3. Effect of various materials on the determination of OxLDL. OxLDL (10 ng) mixed with 1 μg (hatched bar) or 10 μg (shaded bar) of materials indicated under the abscissa was added to the microtiter well precoated with the monoclonal antibody FOH1a/DLH3. Then the sandwich ELISA was performed as described in the legend of Fig. 1. Values are mean ± standard deviation of three experiments. BSA: bovine serum albumin, HSA: human serum albumin, FGN: fibrinogen, FN: fibronectin, VN: vitronectin, Hepa-S: heparan sulfate, Chondr-S: chondroitin sulfate.

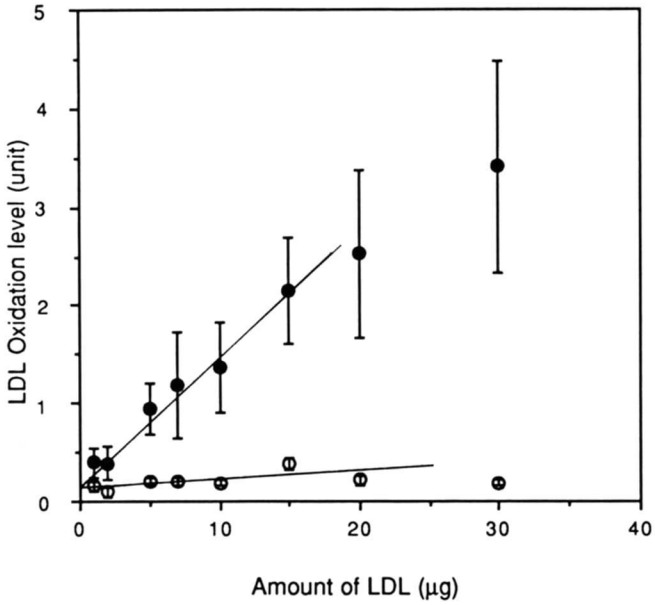

Fig. 4. Reactivity of native LDL from healthy subjects as determined by the sandwich ELISA. Microtiter wells were precoated with the monoclonal antibody FOH1a/DLH3 (closed circle) or nonimmune murine IgM (open circle). To these wells 1 to 30 μg protein of LDL was added and the assay was carried out as described in the legend of Fig. 1. Values are mean ± standard deviation of four individual samples. One unit of oxidation level represents the reactivity equal to 1 ng of standard OxLDL.

proteins from plasma or vessel walls would have little effect on the assay.

Measurement of human plasma LDL oxidation levels

In the sandwich ELISA assay, when large amounts of native LDL from healthy subjects were added to microtiter wells instead of OxLDL, the absorbance increased in a dose-dependent manner up to 15 μg (**Fig. 4**). Some absorbance was also observed when nonimmune murine IgM was precoated onto the microtiter wells instead of FOH1a/DLH3. As micrograms of LDL were charged to the wells, a very small amount of LDL may bind to murine IgM or the surface of plastic plates nonspecifically. It may also be due to some minor components of the LDL preparations. Therefore, parallel measurements of LDL oxidation levels were made using FOH1a/DLH3 and nonimmune murine IgM for each sample and the total value was corrected to compensate for the absorbance due to the nonspecific reaction. There was significant reactivity in native LDL even after subtracting the background with nonimmune IgM, suggesting that the native LDL fraction might contain a low level of LDL oxidation. The reactivity was not changed after the native LDL fraction and the original plasma were stored in refrigerator for a week in the presence of EDTA (data not shown).

1-Palmitoyl-2-linoleoyl PC oxidized by ferrous ion and ascorbate in the presence of BSA was subjected to a G-3000SWXL gel permeation HPLC column to separate OxPC-BSA complex from free OxPC molecules and remaining PC liposomes (**Fig. 5A**). When aliquots of this OxPC-BSA complex fraction were added to the sandwich ELISA, the reactivity of LDL was inhibited; untreated BSA was not inhibitory (Fig. 5B). This result suggests that the reactivity in native LDL was obtained through the specificity of FOH1a/DLH3. Addition of OxPC to native LDL, however, greatly increased the response (data not shown). It is very likely that association of OxPC molecules with LDL particles, either by chemical modification on apoB protein or by transfer-

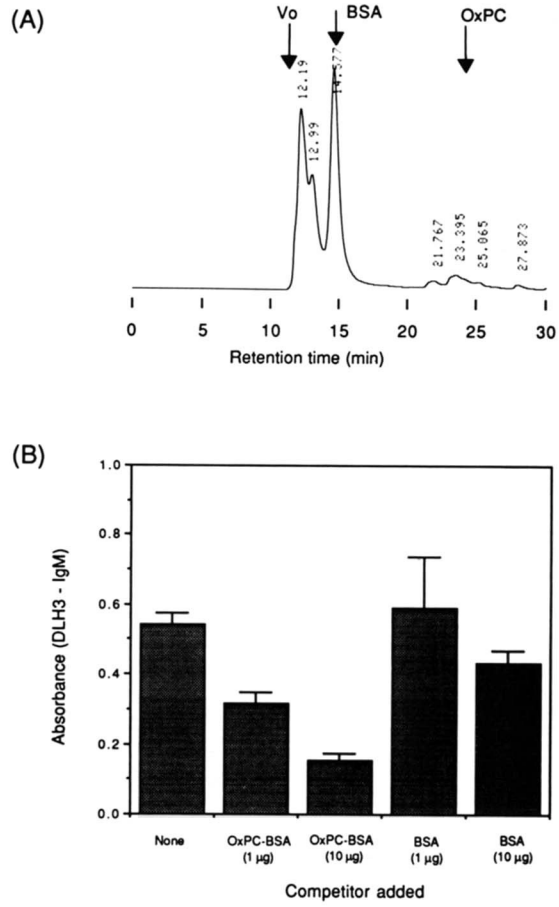

Fig. 5. Inhibition of the reactivity in normal plasma LDL fraction by OxPC-BSA complex. (A) 1-Palmitoyl-2-linoleoyl PC was incubated with ascorbic acid and $FeSO_4$ in the presence of BSA (0.2 mg/ml) at 37°C for 3 h. The reaction mixture was subjected to a G-3000SWXL gel permeation HPLC column. The absorbance at 220 nm was monitored. The peak corresponding to that of BSA standard was recovered. Arrows indicate the void volume and the peak of BSA respectively. (B) OxPC-BSA (3 μg) was added to the microtiter well precoated with the monoclonal antibody FOH1a/DLH3. Subsequently, 10 μl of LDL (20 μg) was added to the well. The sandwich ELISA was performed as described in the legend of Fig. 1. Values are mean ± standard deviation of four experiments.

TABLE 1. Plasma LDL oxidation levels in normal, hyperlipidemic, and hemodialytic subjects

	Normal (n = 9)	Hemodialysis (n = 16)
Age, yr	29.3 ± 4.9	57.7 ± 12.2
LDL oxidation level, units/5 μg LDL protein	0.52 ± 0.35	4.19 ± 2.59
Total plasma cholesterol, mg/dl	165 ± 34	165 ± 61

LDL oxidation levels and total cholesterol of normal and hemodialytic patients plasma were determined as described in Materials and Methods. Briefly, after the LDL fraction was separated from each plasma, LDL (5 μg/well) was added to microtiter wells precoated with the monoclonal antibody or nonimmune murine IgM. One unit of LDL oxidation level represents the reactivity corresponding to 1 ng of standard OxLDL. Values are mean ± standard deviations.

ring to lipophilic atmosphere of LDL, forms antigenic OxPC-LDL complex.

We applied this method for the measurement of oxidation levels in LDL fractions to both normal subjects and hemodialytic patients (**Table 1**). We decided to measure the reactivity in 5 μg protein of LDL from each subject so that the results could be directly compared under the same assay conditions. The results were expressed as "LDL oxidation level" using an oxidation unit that was defined as the reactivity equal to 1 ng of copper-induced OxLDL standard. The mean and standard deviation of the oxidation level in 5 μg protein of LDL from normal subjects was 0.52 ± 0.35 units (n = 9). Surprisingly, the LDL oxidation level of the patients receiving hemodialysis was 4.19 ± 2.59 units (n = 16), about eightfold higher than in normal plasma. There was no significant difference between total plasma cholesterol levels of the patients and normal subjects. Heparin, which was injected into the patients during hemodialysis, did not affect the recovery of OxLDL in the sandwich ELISA, up to 1 IU/well (data not shown).

After human plasma was ultracentrifuged on a NaBr density gradient, lipoprotein-deficient plasma (LPDP) was recovered from the bottom fractions of density >1.22 g/ml. When aliquots of plasma and LPDP fractions were subjected to the sandwich ELISA, significant absorbance was observed (**Fig. 6**). The volumes of LDL and LPDP fractions recovered from 10 ml of plasma were 7 ml and 14 ml, respectively. These samples were diluted by this ratio so that each set of sample should be equivalent to 5 μg of LDL. The reactivity found in LPDP seemed to be apoB-independent, as almost the same reactivity was observed without the anti-apoB antibody. Furthermore, the reactivity varied extremely among donors. Although, as yet, we do not know what the reactive materials in LPDP are, this nonspecific reaction may be caused by ALP activities in plasma or by unknown components causing linkage of the monoclonal antibody and the ALP-conjugated second antibody. The reactivity in plasma was partially reduced by omitting anti-apoB antibody from the procedure. As the reactivity in the LDL fractions was certainly dependent on the presence of anti-apoB antibody, we decided to use the LDL fraction rather than whole plasma to measure LDL oxidation levels.

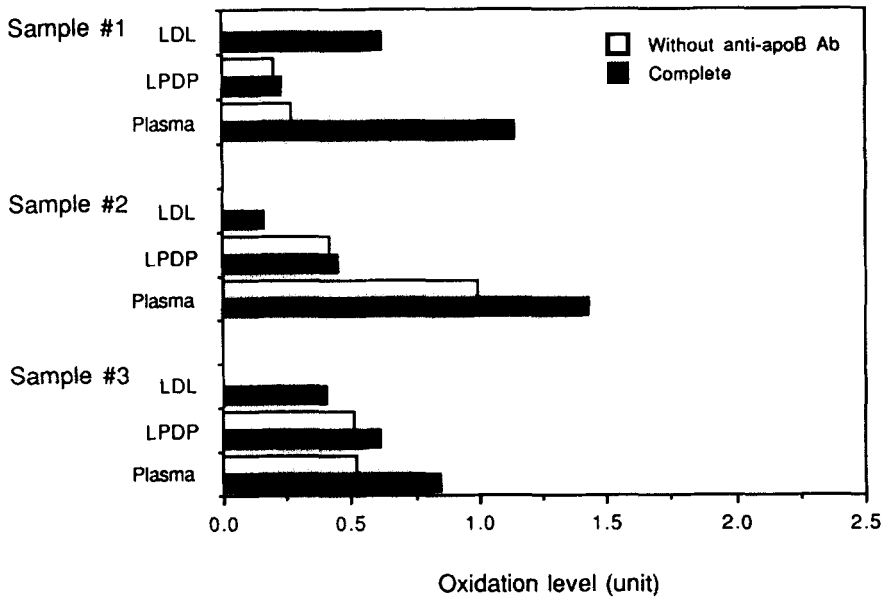

Fig. 6. Comparison of the reactivities in native LDL, LPDP, and whole plasma from healthy subjects as determined by the sandwich ELISA. To the microtiter wells precoated with the monoclonal antibody FOH1a/DLH3 or nonimmune murine IgM, LDL (5 μg) as well as the equivalent amount of LPDP and plasma from four healthy subjects were added. The assay was carried out with (closed bar) or without (open bar) incubating with anti-apoB antibody. Results were expressed as oxidation units in the samples.

DISCUSSION

In this study we have developed a new method to measure very low concentrations of OxLDL using an anti-OxLDL monoclonal antibody. As little as 0.5 ng of copper-induced OxLDL was detected by introducing a sandwich ELISA procedure, and this increase in sensitivity enabled us to detect very low levels of LDL oxidation present in the native LDL fraction of human plasma.

OxLDL has been implicated in the initiation and promotion of atherosclerosis (9–11). Lipid-laden foam cells were formed from macrophages by taking up OxLDL through several receptors (12, 13, 28, 29). OxLDL also showed various pathophysiological activities, including enhancement of interaction between leukocytes and endothelial cells (14–18). The relationship between atherogenicity and oxidative modification of LDL has been recognized for some time. A number of investigators have measured TBA reactive substances in plasma and lipid hydroperoxides using HPLC; although these methods suggest lipid peroxidation they do not measure the formation of oxidatively modified LDL directly.

The specificity of the assay used in this study is largely due to the specificity of the monoclonal antibody FOH1a/DLH3 (20). This antibody does not react with native, acetylated, or malondialdehyde-treated LDL. The epitope of the antibody was shown to be OxPC product(s) that form complexes with apoB protein of the LDL particle. Further study on the isolation and structural analysis of this compound(s) is currently being carried out.

Choice of an appropriate standard material is very important to ensure a reproducible assay. We used copper-induced OxLDL prepared under controlled conditions as a standard, as there is as yet no good established standard. It is very important to control the conditions under which OxLDL is prepared. When the structure of this antibody's epitope (OxPC products) is eventually clarified, it may be important to use the antigenic OxPC molecule(s) or their derivatives as standards and hence ensure the quantitativeness of the method.

OxLDL is a mixture of very heterogeneous products of lipoprotein modification. An LDL particle can be modified in a variety of ways, with the production of OxPC being one of those. The antigenic materials detected by this method are defined as apoB-containing particles associated with OxPC. It should be noted that it is difficult to express quantitatively the amount of oxidatively modified LDL in human plasma LDL fractions because the reactivities of an OxLDL particle in this assay would be affected by the degree of apoB and the number of OxPC molecules associated to apoB protein. In this study we decided to represent the results using an oxidation unit by comparing with the reactivity of the standard OxLDL to the antibodies.

The determinant of this new method is not only unique but also pathologically interesting, as the monoclonal antibody detected macrophage-derived foam cells in human atherosclerotic lesions but antigen was not observed in the extracellular space of these foam cells (20). MDA-conjugated LDL and 4-hydroxynonenal-conjugated LDL have been cited in a number of previous studies as examples of oxidatively modified LDL. They have been detected immunologically in atherosclerotic lesions of WHHL aortas, although the localization of the antigens within the lesions seemed diffuse in some cases (10, 30, 31).

It is noteworthy that it would be easy to adapt this method to determine other oxidized lipoproteins or oxidatively modified components by replacing the anti-apoB antibody with other antibodies.

Very recently Kotani et al. (32) reported a sandwich ELISA method to determine MDA-LDL using two monoclonal antibodies against MDA and human apoB. They detected high levels of MDA-LDL (17.1–50.2 μg/ml) in healthy human sera. Because our monoclonal antibody FOH1a/DLH3 does not react with MDA-LDL at all (20), the activity we detected was different from MDA-LDL. It is possible that MDA-LDL may be present in human blood at a higher concentration than OxPC-modified LDL.

While measuring plasma LDL oxidation levels, it is important to keep in mind possible interference by other components of plasma. When lipoprotein-deficient plasma was subjected to the assay, we observed a small, but significant, absorbance (Fig. 6). This activity was independent of anti-apoB antibody, suggesting that some unknown components interacted with the immunoglobulins. The LDL oxidation levels in plasma might be directly measured by taking the difference between its reactivities in the presence and absence of the anti-apoB antibody. The apoB-dependent absorbance obtained for diluted plasma, however, did not show good correlation to that of the corresponding LDL. For this reason we considered it better to separate the LDL fraction from plasma before carrying out the assay in this study. Even the LDL fraction seemed to show some interference by nonspecific interaction, as some background absorbance was observed using nonimmune IgM instead of FOH1a/DLH3 (Fig. 4). It is therefore necessary to correct for this by subtracting the nonspecific absorbance of nonimmune IgM from the value of FOH1a/DLH3. It would certainly be useful if the oxidation levels in plasma could be measured without separating LDL, although further experiments are needed to elucidate a reliable assay procedure for this.

The level of OxLDL detected in LDL from hemodialysis patients was much higher than that of normal subjects. This observation suggests three points of interest. First, this method is sensitive enough to detect changes in LDL oxidation levels in human plasma, enabling it to be used with human samples.

Second, under some clinical conditions LDL is oxidized and the modified LDL is present in circulating blood. The LDL oxidation level detected in normal subjects was 0.52 ± 0.35 units/5 μg protein of LDL. It has been postulated that the oxidative modification of LDL takes place in the vessel wall (7), although the mechanism of LDL oxidation in vivo is not yet clear. Our present data, however, raise an alternative possibility that OxLDL may, at least in part, be generated or released in circulating blood. Recently autoantibodies to MDA-LDL were detected frequently in healthy human sera (33, 34). Detection of MDA-LDL in human serum by a sandwich ELISA was also reported (32). These observations also support the possible presence of oxidatively modified LDL in blood flow.

Third, renal dysfunction may be related to in vivo oxidation of LDL. In this study we did not obtain sufficient data to elucidate the effects of various factors such as age, complications, and types of hemodialysis membranes on the LDL oxidation level. We also do not know why the hemodialysis patients had increased plasma LDL oxidation levels. It is, however, well known that hyperlipidemia often accompanies renal diseases (6). Lipid-laden cells, similar to the foam cells in atherosclerotic lesions, have been observed in glomeruli in experimental nephrotic animals (35, 36). Materials related to oxidized fatty acids were also detected in the glomerulosclerotic lesions (36), although the mechanism for foam cell formation in glomeruli has not been clarified. Increased levels of TBA reactive substances in plasma of patients treated with maintenance hemodialysis have been observed (37). Recently it was reported that LDL from these patients showed a longer lag time against copper-induced oxidation and a higher UV absorbance for conjugated dienes than normal LDL (38), suggesting that the patients' LDL had already been significantly oxidized. Another possibility is that the hemodialysis treatment itself might cause oxidative modification of LDL through activation of neutrophils by contact with dialysis membranes (39, 40). However, it is still controversial whether the activation of neutrophils during hemodialysis results in lipid peroxidation in plasma (39).

Further study is certainly necessary to investigate the pathophysiological significance of OxLDL. Our new method would be a useful tool to investigate the involvement of OxLDL in certain pathological conditions and may have diagnostic implications as well.

The authors thank the hemodialysis treatment team of Kimitsu Hospital for providing patients' plasma. We also thank Dr. Kumiko Nakajima of Kyorin University and Ms. Akemi Uchida of Teikyo University for their assistance. This work was supported in part by Research Fund from Kanagawa Academy of Science and Technology (No. 94008) and from grant-in-aid for Scientific Research from Ministry of Education, Science and Culture of Japan (Nos. 06772149 and 06454602).

Manuscript received 12 May 1995 and in revised form 25 October 1995.

REFERENCES

1. Steinbrecher, U. P., S. Parthasarathy, D. S. Leake, J. L. Witztum, and D. Steinberg. 1984. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc. Natl. Acad. Sci. USA.* **81:** 3883–3887.
2. Carew, T. E., D. C. Schwenke, and D. Steinberg. 1987. Antiatherogenic effect of probucol unrelated to its hypocholesterolemic effect: evidence that antioxidants in vivo can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of highperlipidemic rabbit. *Proc. Natl. Acad. Sci. USA.* **84:** 7725–7729.
3. Sasaki, T., T. Tanishima, T. Asano, Y. Mayanagi, and K. Sano. 1979. Significance of lipid peroxidation in the genesis of chronic vasospasm following rupture of an intracranial aneurysm. *Acta Neurochir. (Suppl.)* **28:** 536–540.
4. Yoshikawa, T., K. Yamaguchi, M. Kondo, N. Mizukawa, T. Ohta, and K. Hirakawa. 1982. Thiobarbituric acid reactive substances and vitamin E in serum and cerebrospinal fluid from patients with cerebral apoplexy. *Arch. Gerontol. Geriatr.* **1:** 209–218.
5. Fillit, H., E. Elion, J. Sullivan, R. Sherman, and J. B. Zabriskie. 1981. Thiobarbituric acid reactive materials in uremic blood. *Nephron.* **29:** 40–43.
6. Gröne, E. F., A. K. Walli, H-J. Gröne, B. Miller, and D. Seidel. 1994. The role of lipids in nephrosclerosis and glomerulosclerosis. *Atherosclerosis.* **107:** 1–13.
7. Sato, Y., N. Hotta, N. Sakamoto, S. Matsuoka, N. Ohishi, and K. Yagi. 1979. Lipid peroxide level in plasma of diabetic patients. *Biochem. Med.* **21:** 104–107.
8. Asayama, K., H. Hayashibe, K. Dobashi, T. Niitsu, A. Miyao, and K. Kato. 1989. Antioxidant enzyme status and lipid peroxidation in various tissues of diabetic and starved rats. *Diabetes Res.* **12:** 85–91.
9. Witztum, J. L., and D. Steinberg. 1991. Role of oxidized low density lipoprotein in atherogenesis. *J. Clin. Invest.* **88:** 1785–1792.
10. Palinski, W., M. E. Rosenfeld, S. Ylä-Herttuala, G. C. Gurtner, S. S. Socher, S. W. Butler, S. Parthasarathy, T. E. Carew, D. Steinberg, and J. L. Witztum. 1989. Low density lipoprotein undergoes oxidative modification in vivo. *Proc. Natl. Acad. Sci. USA.* **86:** 1372–1376.
11. Rosenfeld, M. E., J. C. Khoo, E. Miller, S. Parthasarathy, W. Palinski, and J. L. Witztum. 1991. Macrophage-derived foam cells freshly isolated from rabbit atherosclerotic lesions degrade modified lipoproteins, promote oxidation of low-density lipoproteins, and contain oxidation-specific lipid-protein adducts. *J. Clin. Invest.* **87:** 90–99.

12. Naganuma, S., N. Kuzuya, K. Sakai, K. Hasumi, and A. Endo. 1992. Inhibition of the accumulation of lipid droplets in macrophage J774 by bafilomycin B_1 and destruxin E. *Biochim. Biophys. Acta.* **1126:** 41–48.
13. Lougheed, M., H. Zhang, and U. P. Steinbrecher. 1991. Oxidized low density lipoprotein is resistant to cathepsins and accumulates within macrophages. *J. Biol. Chem.* **266:** 14519–14525.
14. Lehr, H. A., C. Hübner, B. Finckh, S. Angermüller, D. Nolte, U. Beisiegel, A. Kohlschütter, and K. Messmer. 1991. Role of leukotriens in leukocyte adhesion following systemic administration of oxidatively modified human low density lipoprotein in hamsters. *J. Clin. Invest.* **88:** 9–14.
15. Jeng, J-R., C-H. Chang, S-M. Shieh, and H-C. Chiu. 1993. Oxidized low density lipoprotein enhances monocyte-endothelial cell binding against shear-stress-induced detachment. *Biochim. Biophys. Acta.* **1178:** 221–227.
16. Murugesan, G., G. M. Chisolm, and P. L. Fox. 1993. Oxidized low density lipoprotein inhibits the migration of aortic endothelial cells in vitro. *J. Cell Biol.* **120:** 1011–1019.
17. Boulanger, C. M., F. C. Tanner, M-L. Béa, A. W. A. Hahn, A. Werner, and T. F. Lúscher. 1992. Oxidized low density lipoproteins induce mRNA expression and release of endothelin from human and porcine endothelium. *Circ. Res.* **70:** 1191–1197.
18. Martin-Nizard, F., H. S. Houssaini, S. Lestavel-Delattre, P. Duriez, and J-C. Fruchart. 1991. Modified low density lipoproteins activate human macrophages to secrete immunoreactive endothelin. *FEBS Lett.* **293:** 127–130.
19. Ylä-Herttuala, S., W. Palinski, M. E. Rosenfeld, S. Parthasarathy, T. E. Carew, S. Butler, J. L. Witztum, and D. Steinberg. 1989. Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *J. Clin. Invest.* **84:** 1086–1095.
20. Itabe, H., E. Takeshima, H. Iwasaki, J. Kimura, Y. Yoshida, T. Imanaka, and T. Takano. 1994. A monoclonal antibody against oxidized lipoprotein recognizes foam cells in atherosclerotic lesions. Complex formation of oxidized phosphatidylcholines and polypeptides. *J. Biol. Chem.* **269:** 15274–15279.
21. Redgrave, T. G., D. C. K. Roberts, and C. E. West. 1975. Separation of plasma lipoproteins by density-gradient ultracentrifugation. *Anal. Biochem.* **65:** 42–49.
22. Bronzert, T. J., and H.B. Brewer, Jr. 1977. New micromethod for measuring cholesterol in plasma lipoprotein fractions. *Clin. Chem.* **23:** 2089–2098.
23. Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150:** 76–85.
24. Abele, J., and H. Khayam-Bashi. 1979. Aqueous primary standard for use in measuring cholesterol by the cholesterol oxidase method. *Clin. Chem.* **25:** 132–135.
25. Buege, J. A., and S. D. Aust. 1978. Microsomal lipid peroxidation. *Methods Enzymol.* **52:** 303–310.
26. Noble, R. P. 1968. Electrophoretic separation of plasma lipoproteins in agarose gel. *J. Lipid Res.* **9:** 693–700.
27. Zhou, X., and G. Arthur. 1992. Improved procedures for the determination of lipid phosphorus by malachite green. *J. Lipid Res.* **33:** 1233–1236.
28. Freeman, M., Y. Ekkel, L. Rohrer, M. Penman, N. J. Freedman, G. M. Chisolm, and M. Krieger. 1991. Expression of type I and type II bovine scavenger receptors in Chinese hamster ovary cells: lipid droplet accumulation and nonreciprocal cross competition by acetylated and oxidized low density lipoprotein. *Proc. Natl. Acad. Sci. USA.* **88:** 4931–4935.
29. Endemann, G., L. W. Stanton, K. S. Maddem, C. M. Bryant, R. T. White, and A. A. Protter. 1993. CD36 is a receptor for oxidized low density lipoprotein. *J. Biol. Chem.* **269:** 11811–11816.
30. Haberland, M. E., D. Fong, and L. Cheng. 1988. Malondialdehyde-altered protein occurs in atheroma of Watanabe heritable hyperlipidemic rabbits. *Science.* **214:** 215–218.
31. Rosenfeld, M. E., W. Palinski, S. Ylä-Herttuala, S. Butler, and J. L. Witztum. 1990. Distribution of oxidation specific lipid-protein adducts and apolipoprotein B in atherosclerotic lesions of varying severity from WHHL rabbits. *Arteriosclerosis.* **10:** 336–349.
32. Kotani, K., M. Maekawa, T. Kanno, A. Kondo, N. Toda, and M. Manabe. 1994. Distribution of immunoreactive malondialdehyde-modified low-density lipoprotein in human serum. *Biochim. Biophys. Acta.* **1215:** 121–125.
33. Parums, D. V., D. L. Brown, and M. J. Mitchinson. 1990. Serum antibodies to oxidized low-density lipoprotein and ceroid in chronic periaortitis. *Arch. Pathol. Lab. Med.* **114:** 383–387.
34. Salonen, J. T., S. Ylä-Herttuala, R. Yamamoto, S. Butler, H. Korpela, R. Salonen, K. Nyyssönen, W. Palinski, and J. L. Witztum. 1992. Autoantibody against oxidised LDL and progression of carotid atherosclerosis. *Lancet.* **339:** 883–887.
35. van Goor, H., M. L. C. van der Horst, J. Atmossoerodjo, J. A. Joles, A. van Tol, and J. Grond. 1993. Renal apolipoproteins in nephrotic rats. *Am. J. Pathol.* **142:** 1804–1812.
36. Magil, A. B., J. J. Frohlich, S. M. Innis, and U. P. Steinbrecher. 1993. Oxidized low-density lipoprotein in experimental focal glomerulosclerosis. *Kidney Int.* **43:** 1243–1250.
37. Schettler, V., E. Wieland, R. Verwiebe, P. Schuff-Werner, F. Scheler, and M. Oellerich. 1994. Plasma lipids are not oxidized during hemodialysis. *Nephron.* **67:** 42–47.
38. Loughrey, C. M., I. S. Young, J. McEnerry, I. F. W. McDowell, C. McMaster, P. T. McNamee, and E. R. Trimble. 1994. Oxidation of low density lipoprotein in patients on regular haemodialysis. *Atherosclerosis.* **110:** 185–193.
39. Zochée, P., R. Daelemans, P. Pollaris, M. A. Boogaerts, and R. L. Lins. 1994. Neutrophil adhesion molecules in chronic hemodialysis patients. *Nephron.* **68:** 192–196.
40. Combe, C., M. Pourtein, V. de Précigout, A. Baquey, D. Morel, L. Potaux, P. Vincendeau, J. H. Bézian, and M. Aparicio. 1994. Granulocyte activation and adhesion molecules during hemodialysis with cuprophane and a high-flux biocompatible membrane. *Am. J. Kidney Dis.* **24:** 437–442.