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Polyclonal Antibodies against Formaldehyde-modified Apolipoprotein A-I

An Approach to Circumventing Fixation-Induced Loss of Antigenicity in Immunocytochemistry

Bärbel Harrach and Horst Robenek

A central requirement for the subcellular localization of synthesized and/or endocytosed lipoproteins is the availability of antibodies against the antigen to be localized. The preparation of cells for electron microscopy, in particular the fixation and embedding routine, influences the antigenicity, often resulting in a markedly reduced labeling intensity. To ameliorate fixation-induced changes in antigenicity, we produced antibodies against pre-fixed human apolipoprotein (apo) A-I. Purified apo A-I was fixed with 4% formaldehyde and was used to raise polyclonal antibodies in rabbits. The antiserum was purified by protein A-Sepharose followed by affinity chromatography with the fixed antigen coupled to vinylsulfone-activated agarose. The specificity of the antibodies was ascertained by enzyme-linked immunosorbent assay (ELISA) and Western blot analysis against different fixed and unfixed lipoproteins. Nonspecific binding to unfixed or to fixed apolipoproteins was not observed. Thus, the antibodies reacted specifically with apo A-I and recognized the fixed as well as the unfixed protein. In ELISA, the reaction of the antibodies was markedly enhanced with the fixed antigen, indicating that the antibodies were directed against epitopes characteristically modified by the fixation. The efficacy of the antibodies for light and electron microscopy was tested on HepG2 cells and on human liver cells which are known to synthesize apo A-I. When HepG2 cells were exposed to anti-apo A-I antibodies followed by a secondary fluorescein isothiocyanate-labeled antibody, fluorescence was found intracellularly in distinct regions. Electron microscopy revealed that the endoplasmic reticulum, and in particular the trans elements of the Golgi complexes, were the main compartments stained for apo A-I both in HepG2 cells, as shown by the immunoperoxidase technique, and in human hepatocytes, as shown by the protein A-gold technique on ultrathin cryosections. Furthermore, we were able to localize apo A-I in foam cells of the human aortic plaque by postembedding immunolabeling techniques. These findings demonstrate the potential of antibodies to fixed lipoproteins in impaired localization at the light microscopic and electron microscopic levels. (Arteriosclerosis 10:564-576, July/August 1990)

Apolipoprotein (apo) A-I is the major protein constituent of plasma high density lipoprotein (HDL), representing about 60% of its protein mass. In humans, apo A-I is primarily synthesized by intestinal cells and liver cells and is secreted as a basic precursor protein, which is subsequently processed to more acidic isoproteins found in plasma HDL.¹⁻⁶ Some studies suggest that apo A-I may also function as a ligand for HDL binding to cells.⁷⁻¹² The inverse relationship of HDL to apo A-I levels and the incidence of coronary artery disease¹³ led to our interest in apo A-I metabolism. Several biochemical and morphological techniques with immunoassays¹⁴⁻¹⁷ were used to quantify and localize apo A-I.

However, attempts to localize apo A-I by immunoelectron microscopy in cells have so far failed or given unsatisfactory results. The reason for this lack of success is presumably related to the preparation procedures used for electron microscopy. In particular, the fixation routine often adversely influences antigenicity, resulting in a markedly reduced labeling intensity. Formaldehyde, regarded as a "light" fixative and often used for immunoelectron microscopy, is known to react with amino groups of proteins and to cross-link polypeptide chains, thus leading to impairment or even abolition of immunological reactions.¹⁸⁻²¹

Since it is generally impracticable to avoid these fixation-induced changes of antigenicity, we have tried to improve apo A-I recognition in fixed cells by chemically modifying apo A-I with formaldehyde before immunization. In this way, we hoped to obtain antibodies that were at least in part directed against the antigenic determinants that had been altered by fixation.

To this end, we raised polyclonal antibodies to pre-fixed apo A-I in rabbits and characterized these antibodies with respect to properties relevant to their use in

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morphological studies, especially electron microscopy. The model systems used were human liver cells and human hepatoma HepG2, a cell line known to maintain many of the morphological and biochemical characteristics of normal human hepatocytes, including apo A-I synthesis.^{4,5,22,23,24} The feasibility of applying this approach to atherosclerotic lesions is also illustrated.

Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from Boehringer, Mannheim, F.R.G. Fetal calf serum (FCS), penicillin, streptomycin, sodium pyruvate, L-glutamine, nonessential amino acids, and trypsin ethylenediaminetetraacetate (EDTA) were obtained from GIBCO-BRL, Eggenstein, F.R.G. Protein A-Sepharose CL-4B and a low molecular weight calibration kit were purchased from Pharmacia LKB, Freiburg, F.R.G. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) standards, high range, were from Bio-Rad, Richmond, CA. Divinyl sulfone activated agarose was purchased from Kem-En-Tec, Copenhagen, Denmark. Ampholytes were obtained from Serva, Heidelberg, F.R.G. and decyl sodium sulfate, from Paesel, Frankfurt, F.R.G. Protein A and fluorescein isothiocyanate (FITC)-conjugated goat antirabbit immunoglobulin (IgG) were purchased from Sigma, Munich, F.R.G. Goat antihuman apo A-II was obtained from Boehringer. Rabbit antihuman apo A-I, rabbit antihuman C-II, and antihuman apo C-III were kindly provided by Carsten Ilseemann, Institute for Arteriosclerosis Research, Münster, F.R.G. Peroxidase-conjugated goat antirabbit IgG (H+L)-F(ab')₂ was purchased from Dianova, Hamburg, F.R.G. All other chemicals were of analytical grade and were obtained from Merck, Darmstadt, F.R.G. or Sigma. Additional specific products included tissue culture equipment, Becton Dickinson, Heidelberg, F.R.G.; tissue culture chamber slides, Lab-Tek, Miles, Naperville, IL; Diaflo PM-10 ultrafilters, Amicon, Witten, F.R.G.; polystyrene microtiter plates (high binding capacity), Nunc, Roskilde, Denmark; cellulose nitrate (0.2 µm), Schleicher & Schüll, Dassel, F.R.G.; and Lowicryl K4M, Plano, Marburg, F.R.G.

Preparation and Delipidation of Lipoproteins

Very low density lipoprotein (VLDL) ($d < 1.006$ g/ml), low density lipoprotein (LDL) ($d = 1.019$ to 1.063 g/ml), and HDL ($d = 1.125$ to 1.21 g/ml) were isolated from fresh human serum of individual normolipidemic volunteers by sequential ultracentrifugation²⁵ by using a 50.3 Beckman rotor (Beckman Instruments, Fullerton, CA). The lipoproteins were separated by ultracentrifugation at 49 000 rpm at 4°C for 18 hours (VLDL), 24 hours (LDL), and 48 hours (HDL). For the LDL and HDL preparations, the respective densities were adjusted with potassium bromide. After separation, the lipoprotein fractions were dialyzed against 0.15 M NaCl/5 mM sodium-EDTA/0.01% sodium azide, pH 7.4, at 4°C. The lipoproteins were stored at 4°C and were used within 5 days after preparation.

For isoelectric focusing (IEF), HDL samples were delipidated successively with two separate extractions of

40 volumes of ethanol/diethylether (3:1, vol/vol) for 15 hours at -20°C each and with diethylether for 1 hour at -20°C. The apo HDL were solved in 0.01 M of Tris-HCl, pH 8.2, and were incubated for 30 minutes at 37°C. The remaining diethylether was removed under a stream of nitrogen. The proteins were stored at -20°C for up to 3 months.

The protein content of the lipoprotein fractions was determined by a modified Lowry method,^{26,27} with bovine serum albumin (BSA) as a standard.

Polyclonal Antibodies to Pre-fixed Apolipoprotein A-I

Purified human apo A-I was kindly provided by Boehringer, Mannheim, F.R.G. For the fixation, 2 mg of the freeze-dried apo A-I was dissolved in 1 ml of phosphate-buffered saline (PBS), pH 7.0, and was mixed with 1 ml of PBS, pH 7.0, containing 8% formaldehyde. Fixation was performed at 4°C for at least 12 hours. Just before application to the rabbits, the apo A-I-formaldehyde solution was concentrated, diluted in 0.9% NaCl, and concentrated again by ultrafiltration (PM-10 filter) under nitrogen. This washing procedure was continued until the estimated formaldehyde concentration was lower than 0.1%, and the protein concentration was about 1 mg/ml 0.9% NaCl.

Male New Zealand White rabbits (3.5 and 4 kg) were immunized by intramuscular injection of 0.4 mg of the pre-fixed apo A-I (fix. apo A-I) in 0.4 ml of 0.9% NaCl containing 100 µl of complete Freund's adjuvant alternately into the left and right hind leg. Immunization was repeated five times at 2-week intervals.

The polyclonal antibodies (IgG) were concentrated by precipitation in 50% saturated ammonium sulfate at room temperature, were dialyzed against PBS, pH 7.0, and were purified by adsorption to protein A-Sepharose. Further purification was done by affinity chromatography. The affinity matrix was prepared by conjugating the fix. apo A-I to divinyl sulfone activated agarose according to the manufacturer's instructions.²⁸ The coupling procedure involved treatment of 7 g of wet gel with 28 mg fix. apo A-I in 17 ml of coupling buffer (1 M of potassium-phosphate buffer, pH 8.6).

Enzyme-linked Immunosorbent Assay

Microtiter plates were coated with 10 ng protein/well in 100 µl of 50 mM sodium carbonate buffer, pH 9.6, for 12 hours at 37°C. Wells were washed four times with PBS and were saturated at 37°C for 1 hour with 200 µl of 1% (wt/vol) BSA in PBS. Plates were washed twice with PBS containing 0.05% Tween 20 (PBST) and were incubated with 100 µl of the diluted antibodies (original protein concentration about 1 mg/ml) for 1 hour at 37°C. After washing four times with PBST, incubation with peroxidase-conjugated goat antirabbit IgG (H+L)-F(ab')₂ (1 hour at 37°C) and washing with PBST, the bound enzyme activity was determined. A mixture of 40 mg of orthophenylenediamine in 100 ml of 0.1 M phosphate-citrate buffer, pH 5.0, containing 0.025% H₂O₂, was used as the enzyme substrate solution. After incubation with the substrate (200 µl) for 30 minutes at 37°C in the dark, the optical density of the reaction product formed was measured at 450 nm.

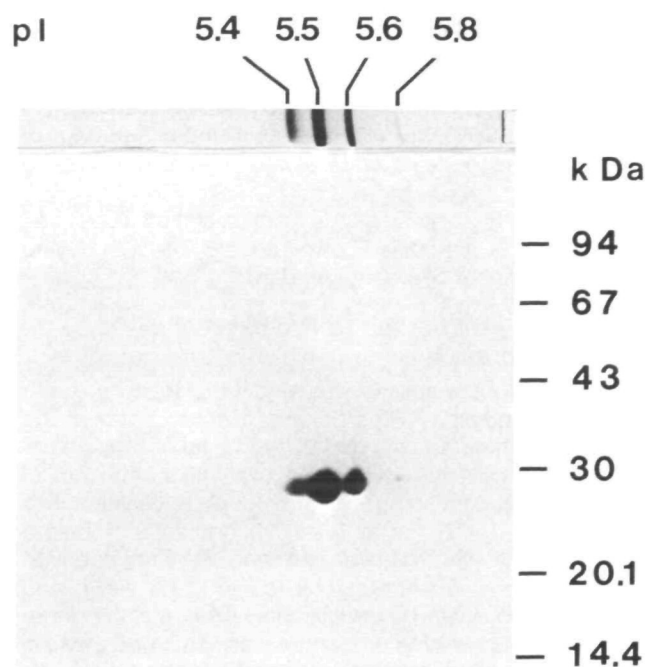


Figure 1. Two-dimensional gel electrophoresis of purified human apolipoprotein A-I. First dimension. Isoelectric focusing (pH 4 to 7). Five micrograms of protein was applied to a 1 mm thick gel. The gel was stained with Coomassie blue R-250. Second dimension. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis 15% acrylamide, silver stained.

Isoelectric Focusing

The apo A-I isoforms, as well as the apoproteins of delipidated HDL, were separated by IEF in a 7.5% polyacrylamide gel containing 8 M urea and 2% ampholytes (pH 4 to 6 or pH 4 to 6 and 5 to 7, one-half by volume). Samples were diluted in 0.01 M of Tris-HCl, pH 8.2, containing 2% ampholytes, 1% decyl sodium sulfate, and 0.1% dithiothreitol. Focusing was performed at 10°C in a vertical electrophoresis chamber (Bio-Rad 220) for 15 hours at 200 V, 1 W, and for 1 hour at 600 V with 20 mM NaOH and 10 mM H₃PO₄ as the cathodic and anodic electrolytes, respectively (Figures 1 and 2) or in a horizontal Multiphor II electrophoresis chamber (Pharmacia LKB) according to the manufacturer's instructions and by using 125×260×0.5 mm gels casted on GelBond films. In the horizontal system, samples were applied about 1 cm from the cathode by using filter paper pieces.

For determination of the pH gradient, slices of the gels (about 1 cm) were cut into 0.5 cm pieces immediately after focusing. After extraction of the gel segments with distilled water for 3 hours at room temperature, the pH values of the solutions were measured. After focusing, gels were either fixed with 15% trichloroacetic acid (TCA) and were stained with Coomassie brilliant blue R-250 or were rapidly frozen and stored until used in two-dimensional gel electrophoresis or electroblotting.

Sodium Dodecyl Sulfate-Polyacrylamide Gradient Gel Electrophoresis and Two-dimensional Gel Electrophoresis

SDS-PAGE on gradient gels of 5% to 15% acrylamide and 3% acrylamide stacking gels was performed under

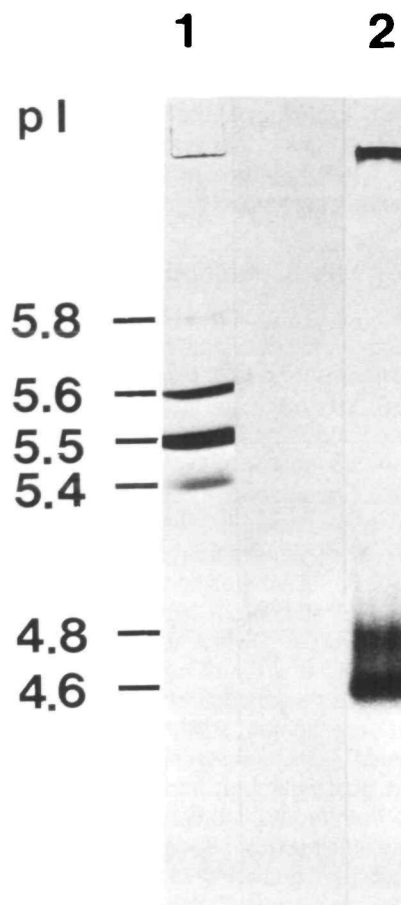


Figure 2. Isoelectric focusing (pH 4 to 7) of unfixed (Lane 1) and fixed (Lane 2) apolipoprotein (apo) A-I. In each lane, 5 µg of apo A-I was applied. Gels were stained with Coomassie blue R-250.

reducing conditions according to the method of Laemmli.²⁹ For molecular weight determination, SDS-PAGE standards (high range) supplemented with bovine erythrocyte carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa), and hen egg white lysozyme (14.3 kDa) were used. After electrophoresis, the gels were fixed with 15% TCA and were stained with Coomassie brilliant blue R-250.

Two-dimensional polyacrylamide gel electrophoresis was performed with IEF in the first, and SDS-PAGE in the second dimension. IEF was performed as described above. The frozen IEF gels were placed in an equilibration solution containing 0.1 M Tris-HCl (pH 6.8), 1% (wt/vol) SDS, and 0.1% (wt/vol) dithiothreitol for 15 minutes at room temperature. SDS-PAGE with a stacking gel (5% T, 3% C) and a separating gel (15% T, 0.7% C) containing 0.1% SDS was performed according to the method of Sprecher et al.³⁰ Low molecular weight standards were used for molecular weight determination. The gels were fixed with 15% TCA and were silver-stained.³¹

Western Blot Analyses

Western blotting was performed after IEF according to the method of Towbin et al.³² Proteins were transferred to nitrocellulose paper (0.2 µm) by using a Nova Blot semi-

dry transfer system (Pharmacia LKB) and a discontinuous buffer system.³³ Running conditions were 0.8 mA/cm² for 1 hour.

The nitrocellulose paper was then cut into strips corresponding to the lanes on the gel. On one strip, protein transfer was verified by a modified India ink stain.^{34,35} The remaining strips were incubated successively with 3% (wt/vol) BSA (2 hours), the primary antibody (4 hours), and the secondary peroxidase-conjugated antibody (2 hours). The incubation buffer for dilution of the antibodies and the several washings consisted of 0.15 M NaCl, 0.05 M Tris-HCl (pH 7.4), and 0.05% Tween 20. Peroxidase activity was revealed by incubation with a substrate solution containing 0.05% chloronaphthol and 0.01% H₂O₂ in incubation buffer for about 4 minutes.

Cell Culture Conditions

Stock cultures of the human hepatoblastoma cell line, HepG2, were kindly provided by Louis Havekes, Gaubius Institute, Leiden, The Netherlands. Cells were grown in DMEM containing 10% FCS, sodium pyruvate (1 mM), L-glutamine (4 mM), nonessential amino acids, penicillin (200 IU/ml), and streptomycin (200 µg/ml) in 75 cm² tissue culture flasks in a humidified incubator at 37°C (5% CO₂). For the experiments, the cells were seeded into 35×10 mm tissue culture dishes or tissue chamber slides and were incubated for 24 hours with the medium mentioned above without FCS.

Fluorescence Microscopy

HepG2 cells grown on chamber slides were fixed for 1 hour at 4°C in PBS (pH 7.4) containing 4% formaldehyde. Cells were washed and permeabilized in PBS containing 0.05% (vol/vol) Nonidet P-40 (incubation buffer) and were incubated with anti-fix. apo A-I (2 µg/ml incubation buffer) for 2 hours at room temperature. After several washings, cells were treated with FITC-conjugated goat antirabbit IgG (dilution 1:50 in incubation buffer) for 1 hour, were washed thoroughly, and were mounted in 90% glycerol in PBS containing 0.1% (wt/vol) paraphenylenediamine. The specificity of immunolabeling was tested by omitting the primary antibody, by using pre-immune serum, or by using the primary antibody after pre-adsorption with the antigen. Fluorescence was viewed with a Zeiss IM 35 fluorescence microscope (450 to 490 nm) and photographed on Kodak Tri-X pan film (400 ASA) with exposure times of about 30 seconds.

Immunoelectron Peroxidase Technique

Cultured HepG2 cells were fixed in McLean and Nakane's fixative³⁶ for 2 to 4 hours at room temperature. After washing and permeabilization in PBS containing 0.05% (wt/vol) saponin and 0.2% gelatine (incubation buffer), cells were incubated with anti-fix. apo A-I (2 µg/ml incubation buffer) and were thoroughly washed. Incubation with the secondary peroxidase-conjugated goat antirabbit IgG (H+L)-F(ab')₂ (1:300) was performed for 1 hour at room temperature. Cells were washed with the buffer defined above and were then fixed for 1 hour at 4°C in 2.5% glutaraldehyde, 5% sucrose, 0.1 M sodium cacodylate buffer (pH 7.4). After intensive washing in 0.05 M

Tris-HCl (pH 7.6), 5% sucrose peroxidase activity was revealed by incubation with 0.05% (wt/vol) 3,3-diaminobenzidine tetrahydrochloride (DAB) in 0.05 M Tris-HCl (pH 7.6) for 10 minutes and 0.01% H₂O₂, 0.05% DAB in 0.05 M Tris-HCl for 3 to 6 minutes. Cells were postfixed with 1.3% OsO₄ in 0.1 M collidine buffer, were dehydrated with alcohol, and were embedded in Epon 812. Thin sections were examined without any further staining in a Philips EM 410 at 60 kV. Control experiments were performed as described in the fluorescence microscopy section.

Immunogold Labeling of Ultrathin Cryosections

Human liver was kindly provided by Peter Zimmer, Children's Clinic, University of Münster, Münster, F.R.G. Tissue blocks (1×1 mm) were fixed with 5% formaldehyde in 0.2 M piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES), pH 7.0, for 2 hours at room temperature. After washing in PIPES buffer, the fixed tissue blocks were infused with a mixture containing 1.6 M sucrose and 25% (wt/vol) polyvinylpyrrolidone (M_w=10 000) and were frozen and mounted on silver pins.³⁷ Ultrathin frozen sections of human liver were cut at -100°C (chamber) and -90°C (knife) with a Reichert-Jung FC 4 E with a diamond knife. Sections were picked up on formvar coated copper grids (150 mesh) and were immunolabeled at room temperature. Immunocytochemical labeling was performed by floating grids serially, section side down, on 5 to 20 µl droplets. The steps were washing with PBS, incubation with anti-fix. apo A-I for 1 hour; washing with PBS, incubation with protein A-gold for 1 hour; and washing with PBS.

The protein A-gold particles were prepared according to the protocol of Roth et al.³⁸ by using gold sols with 15 nm average diameters prepared according to the method of Frens.³⁹

After the labeling procedure, sections were washed intensively with distilled water. The subsequent staining procedure included a 10-minute uranyl acetate oxalate (pH 7.4) stain; three 1-minute rinses in distilled water; and a 10-minute 2% uranyl acetate stain in distilled water. The grids then were floated on drops of 1% tylose in 0.5% uranyl acetate three times for 1 minute each at 0°C.

Controls were performed as described above.

Postembedding Immunocytochemistry of Human Atherosclerotic Lesions

A severe atherosclerotic plaque from a human femoral artery was obtained at surgery and was fixed in McLean and Nakane's fixative.³⁶ This specimen was kindly provided by Eckart Vollmer, Gerhard Domagk Institute of Pathology, University of Münster, Münster, F.R.G. Tissue samples were embedded in Lowicryl K4M⁴⁰ and were orientated to facilitate sectioning of the intima. The presence of abundant foam cells in the intima was confirmed on electron microscopic examination of the sections. Immunolabeling was carried out as described for cryosections above. Sections were stained with uranyl acetate for 10 minutes.

Results

Characterization of the Antigen

To check the purity of the antigen used, human apo A-I was subjected to two-dimensional analysis with IEF (pH 4 to 7) and SDS-PAGE (15%) (Figure 1). As the first dimension IEF gel revealed, purified human apo A-I consisted of four isoforms with isoelectric points (pIs) of 5.8, 5.6, 5.5, and 5.4. The major isoform had a pI of 5.5 and is designated as apo A-I₁. In the second dimension SDS-PAGE, the isoforms of apo A-I were homogeneous, having a molecular weight of about 27 000. No contaminating proteins could be detected by silver staining of the gel.

For characterization of the fixation-induced changes, IEF of the fixed and the unfixed apo A-I was performed (Figure 2). Under the same conditions, IEF of the fixed protein (Lane 2) led to more acidic isoforms. In the Coomassie R-250-stained gel, two protein bands with pIs of 4.8 and 4.6 could be distinguished. In contrast to unfixed apo A-I (Lane 1), focusing of fixed protein did not result in sharp bands but rather in a broad zone ranging from about 4.85 to 4.55.

The occurrence of cross-linking of apo A-I molecules due to formaldehyde fixation was investigated by one-dimensional SDS-PAGE on gradient gels of 5% to 15% acrylamide. It appeared that apo A-I was not highly cross-linked, because most of the proteins had the same molecular weight as the unfixed apo A-I (Figure 3). Only traces of protein were visible at higher molecular weights on the gel. A small amount of apo A-I was cross-linked so that the resulting aggregates could not enter the gel.

Influence of Formaldehyde Fixation on Apolipoprotein A-I Recognition

The influence of formaldehyde fixation on the antigenicity of antibodies to unfixed apo A-I was investigated by enzyme-linked immunosorbent assay (ELISA) by using fixed as well as unfixed apo A-I as the solid phase. Polyclonal antibodies prepared and affinity-purified to the unfixed protein showed a reduced recognition of formaldehyde-treated apo A-I (Figure 4).

Characterization of Polyclonal Antibodies to Formaldehyde-treated Apolipoprotein A-I

Enzyme-linked Immunosorbent Assay

The antibodies raised in rabbits after immunization with the fix. apo A-I were characterized by ELISA by using isolated fixed and unfixed apo A-I, VLDL, LDL, and HDL as the solid phase. Nonsaturating quantities (10 ng) of the apolipoproteins and lipoproteins were used for coating to ensure that comparable amounts of the different proteins bound to the wells. The ELISA data show that the affinity-purified antibodies recognized the fixed, as well as the unfixed, apo A-I, but the former to a much higher extent. Using HDL, the antibody was able to detect apo A-I in HDL molecules, again with a higher affinity to the apo A-I of the fixed lipoprotein. Only slight reactions with VLDL and LDL at high concentration of the antibody was observed (Figure 5). In ELISA, the better recognition of the fixed protein was already clear with unpurified antiserum. The IgG fraction that did not bind to the fix. apo A-I-affinity matrix recognized

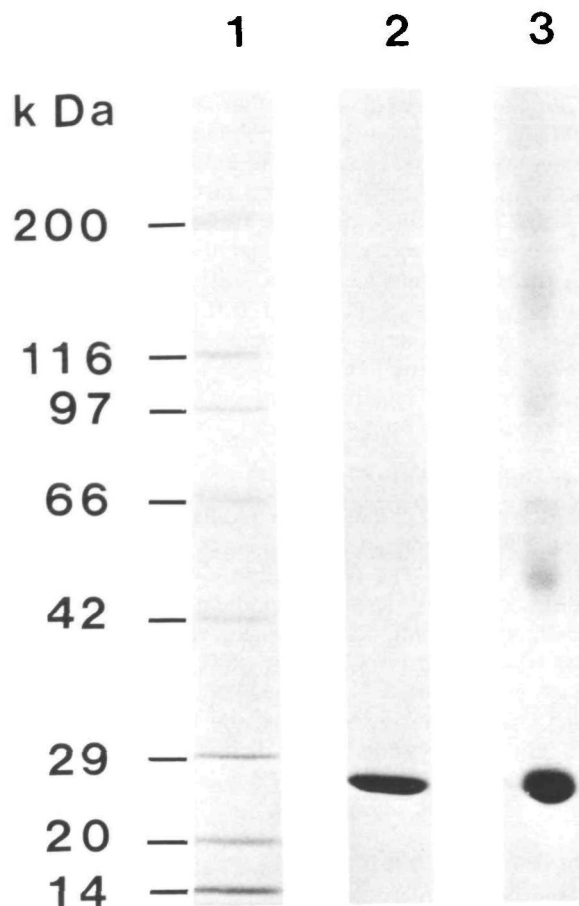


Figure 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis on gradient gels (5% to 15% acrylamide) of unfixed (Lane 2) and fixed (Lane 3) apolipoprotein (apo) A-I. Five micrograms of unfixed and 10 μ g of fixed apo A-I were applied. Molecular weight standards were applied in Lane 1. The gel was stained with Coomassie blue R-250.

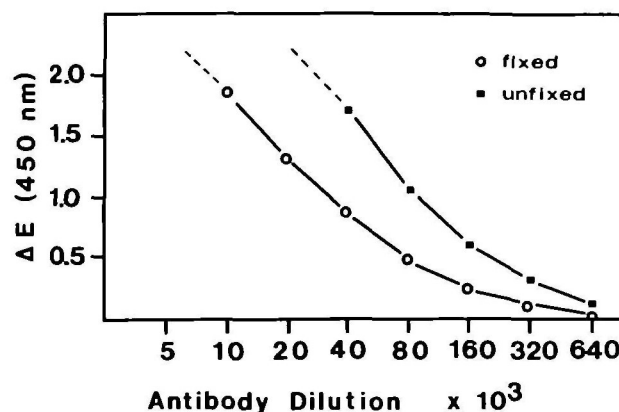


Figure 4. Influence of fixation on apolipoprotein (apo) A-I recognition. Enzyme-linked immunosorbent assay was performed with 10 ng of formaldehyde-fixed or unfixed apo A-I as the solid phase and a dilution series of polyclonal antibodies to unfixed apo A-I. Antibodies showed a decreased recognition of the fixed proteins.

neither fixed nor unfixed apo A-I (data not shown). Control experiments with formaldehyde alone or with formalde-

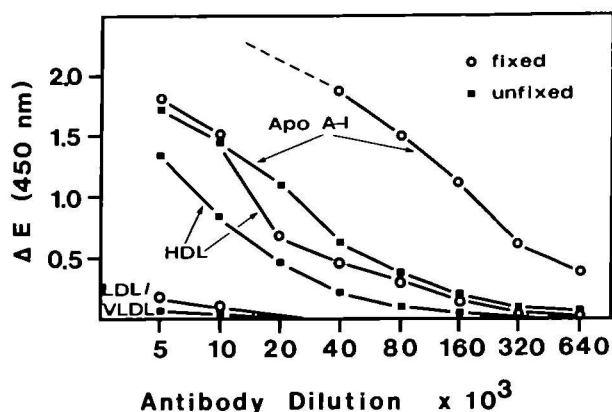


Figure 5. Enzyme-linked immunosorbent assay data gained with a dilution series of anti-fix. apolipoprotein (apo) A-I antibodies. Ten nanograms of fixed or unfixed apo A-I, very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL) were used as the solid phase. Antibodies showed an increased recognition of the fixed proteins.

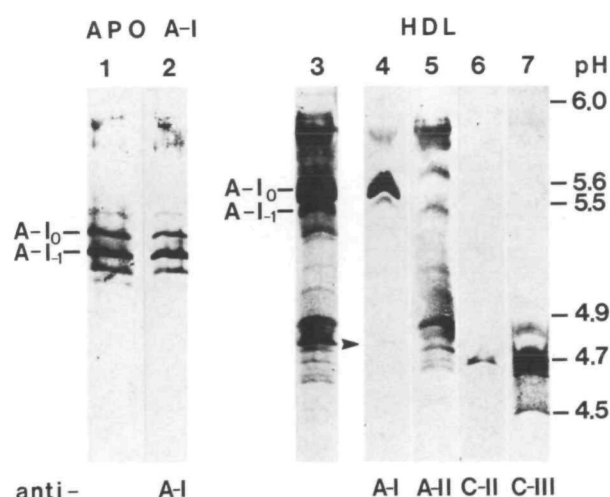


Figure 6. Characterization of antibodies raised against prefixed apolipoprotein (apo) A-I by Western blot analyses. **Lanes 1 and 2.** Electrobots of apo A-I after isoelectric focusing (IEF) (pH 4 to 7). **Lanes 3 to 7.** Electrobots of high density lipoprotein (HDL) after IEF (pH 4 to 6). Five micrograms of apo A-I or 10 μ g of HDL were applied to each lane on 0.5 mm thick gels. **Lanes 1 and 3.** India ink stain of proteins transferred to nitrocellulose. Immunoblot analysis of anti-fix. apo A-I (**Lanes 2 and 4**) was carried out after fixation of the separated isoforms on the nitrocellulose sheet. For the immunoblots of the other antibodies, the fixation was omitted. The antibody dilutions were: anti-fix. apo A-I, 1:20 000; anti-apo A-II, 1:1000; anti-apo C-II and anti-apo C-III, 1:50.

hyde-fixed BSA did not exceed the normal background staining gained with the usual controls.

Western Blot Analyses

To test the isoform and apolipoprotein specificity of the antibody, immunoblotting was performed after IEF of either apo A-I or HDL (Figure 6). As already noted above, fixation changed the pIs of proteins, a fact that complicated the identification of the different apolipoproteins of HDL. Therefore, the proteins were fixed after focusing and were transferred to nitrocellulose directly before the

incubation with anti-fix. apo A-I. Protein transfer to the nitrocellulose sheet was checked by India ink stain (Lanes 1 and 3). As shown in Lane 2, the antibodies recognized all isoforms of the fixed apo A-I. In immunoblots of HDL (Lane 4), the antibody reacted with two isoforms of apo A-I present in HDL. No cross-reactivity to other apolipoproteins of HDL could be observed. In HDL probes of different donors, a slight reactivity to a single additional band was apparent in some cases. From immunoblots performed with anti-apo A-II, C-II, and C-III, (Lanes 5 to 7, respectively), cross-reactivity of the anti-fix. apo A-I with these apolipoproteins could be excluded. In immunoblots with unfixed apo A-I or HDL (data not shown), the antibody showed the same labeling pattern, but with a reduced staining intensity, in agreement with the ELISA data.

In Western blots performed for comparison with antibodies to unfixed apo A-I, reduced recognition of the fixed compared to the unfixed apo A-I was observed.

Localization of Apolipoprotein A-I in HepG2 and Human Liver Cells

Immunofluorescence Staining

The efficacy of the antibody for microscopy was tested on HepG2 cells, which are known to synthesize apo A-I.^{4,5,22,23,24} Using an indirect labeling method with anti-fix. apo A-I and FITC-conjugated goat antirabbit antibodies, the label was found intracellularly in distinct regions (Figures 7A and 7B). Most of the fluorescence was visible as a punctate ring close to and surrounding the nucleus (Figure 7B). In control experiments carried out by using antibodies pre-adsorbed with the antigen (Figure 7C) or by omitting the primary antibody (Figure 7D), no staining was observed.

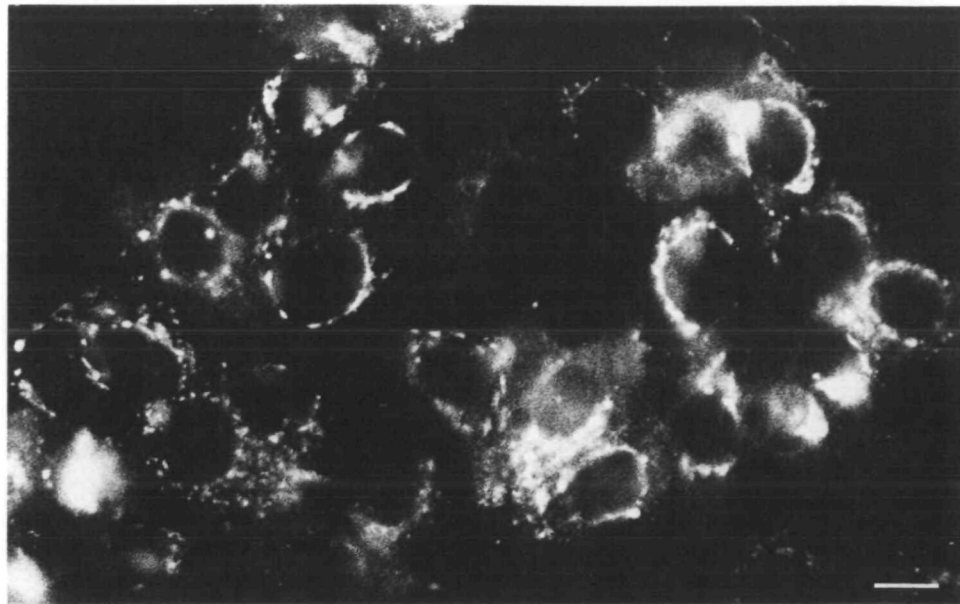
Subcellular Localization

HepG2 Cells

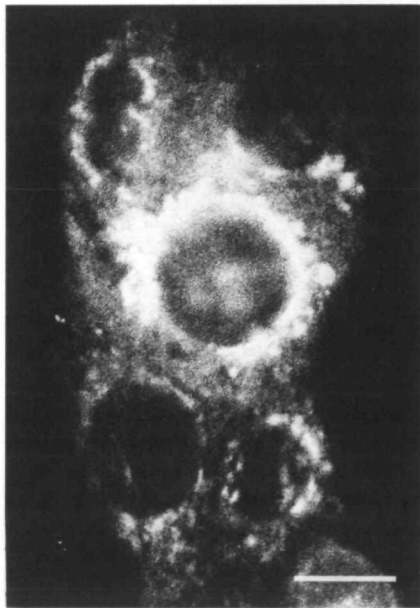
When the antibodies against the prefixed antigen were used, it was possible to visualize apo A-I with the pre-embedding immunoperoxidase technique at the electron microscopic level.

In HepG2 cells, electron microscopy showed dense reaction products localized in the cisternae of the rough endoplasmic reticulum (ER), in the Golgi complexes, and in vesicles associated with the Golgi region, as well as elsewhere in the cell (Figures 8 and 9). There was no positive staining in those organelles in control sections. Other subcellular compartments such as nuclei, mitochondria, and lysosomes were consistently negative.

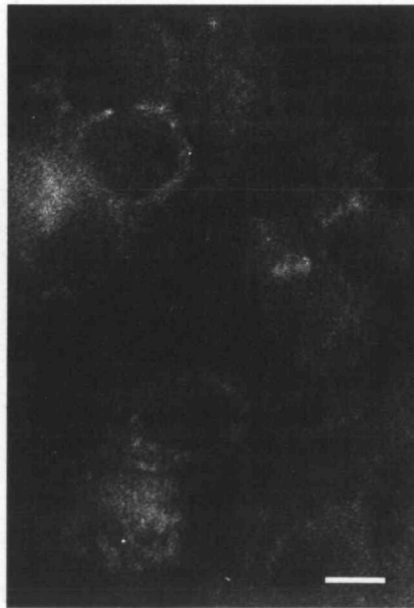
In the rough ER, the membranes and the cisternae were stained, but the ribosomes were not. The staining intensity varied in individual ER lamellae and was often concentrated in the terminal dilations (Figures 8A and 8B). The reaction product that indicated the sites of apo A-I was present in the Golgi lamellae from cis to trans and in the secretion granules on the trans side (Figure 9). On the cis side, vesicular elements of the rough ER were heavily labeled, whereas the two to three innermost Golgi lamellae were weakly stained (Figure 9A). On the trans side of the Golgi complex, many secretion granules with diam-



A



B



C



D

Figure 7. Indirect immunofluorescence staining of apolipoprotein (apo) A-I in HepG2 cells. **A.** Fluorescence staining of apo A-I is present in every HepG2 cell grown in cell culture. $\times 1100$, Bar = $10\ \mu\text{m}$. **B.** Most of the fluorescence label is visible intracellularly in distinct regions close to the nucleus. $\times 1300$, Bar = $10\ \mu\text{m}$. **C.** HepG2 cells incubated with primary antibodies pre-adsorbed with fix. apo A-I are almost devoid of label. **D.** Control experiment carried out by omitting the primary antibody. $\times 800$, Bars = $10\ \mu\text{m}$.

eters of 700 nm showed heavy staining (Figure 9B). Secretion granules similar in their labeling to those located on the trans side of the Golgi complex were seen scattered throughout the cytoplasm.

Human Liver

Antigenic sites for apo A-I were also visualized in the human liver by using postembedding immunocytochemistry performed on ultrathin cryosections in combination with the protein A-gold technique. Antigenic sites for apo A-I were demonstrated in a large number of hepatocytes (Figure 10). The immunoreactivity was

mainly localized in the Golgi area. Positively stained vesicular structures and secretion vesicles were often found in the trans Golgi complex (Figure 10A) and in the pericanalicular region (Figures 10B and 10C). Positive staining was also consistently found in the space of Disse (Figure 10D). The bile canaliculus was completely devoid of label (Figure 10B). In contrast to the localization of the antigen in HepG2 cells with the immunoperoxidase method, in the hepatocytes, gold labeling was found neither in the cisternae of the rough ER nor in those of the Golgi apparatus. No apo A-I was detectable in endothelial, Kupffer, or fat storing cells.

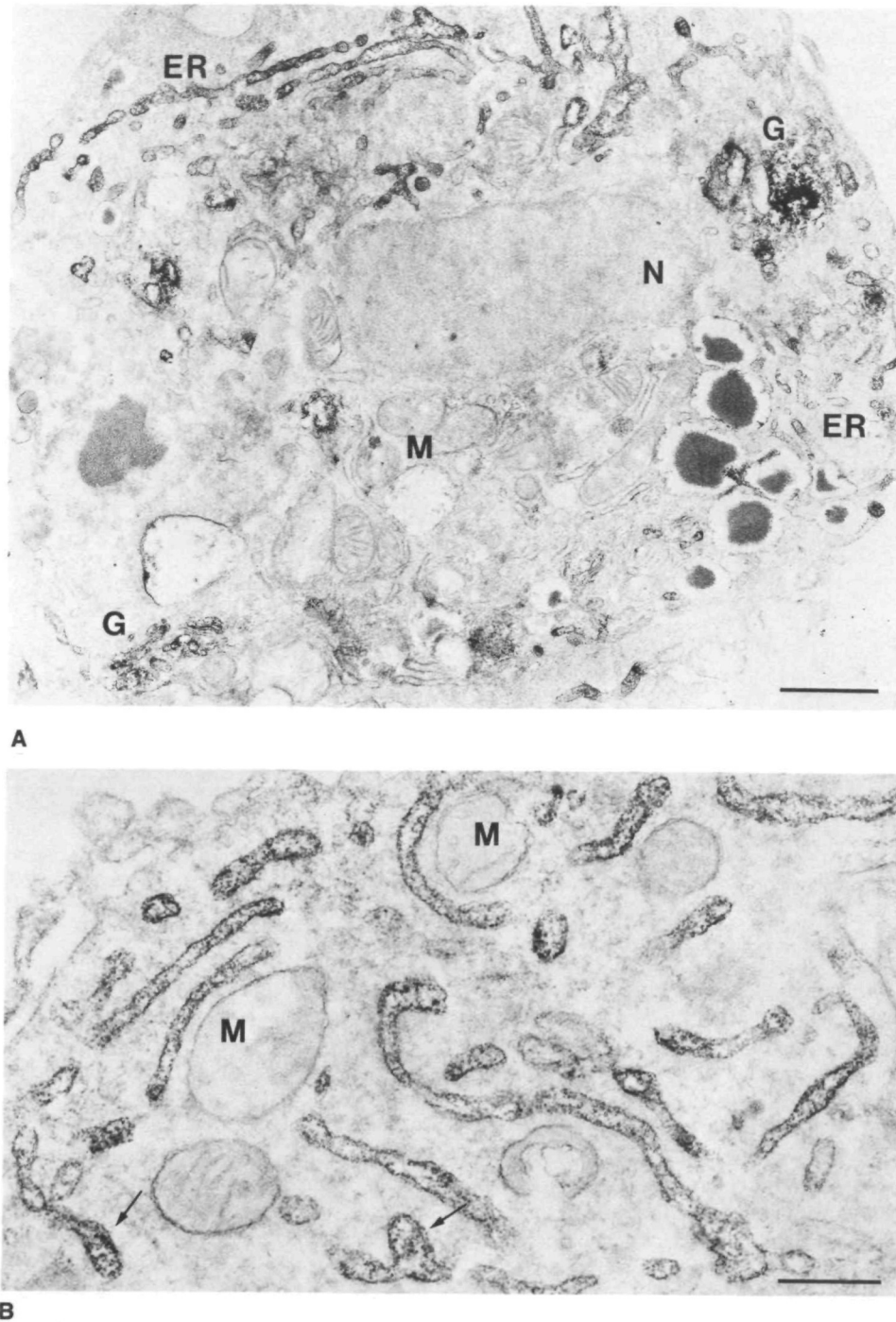


Figure 8. Electron micrographs of HepG2 cells stained by the indirect pre-embedding immunoperoxidase method for apolipoprotein (apo) A-I. **A.** Electron-dense immunoperoxidase reaction products that indicate antigenic sites for apo A-I are present in the cisternae of the rough endoplasmic reticulum (ER), Golgi complexes (G), and secretion vesicles. M=mitochondria, N=nucleus. $\times 18\,600$, Bar=1 μm . **B.** ER-rich region of a HepG2 cell. The staining intensity varies in individual ER lamellae. Some terminal dilatations are heavily stained (arrows). M=mitochondria. $\times 38\,700$, Bar=0.5 μm .

Human Atherosclerotic Lesion

Successful immunolabeling of cells in Lowicryl-embedded human atherosclerotic lesions was also achieved with our antibodies to fix. apo A-I. As illustrated in Figure 11, the cytoplasm of foam cells was most heavily labeled, although lipid droplets and nuclei remained unlabeled. A low level of labeling occurred over

components of the extracellular matrix. Other cell types of the atherosclerotic lesion were devoid of label.

Discussion

One of the major problems of immunoelectron microscopic localization of proteins is the reduction of antigen

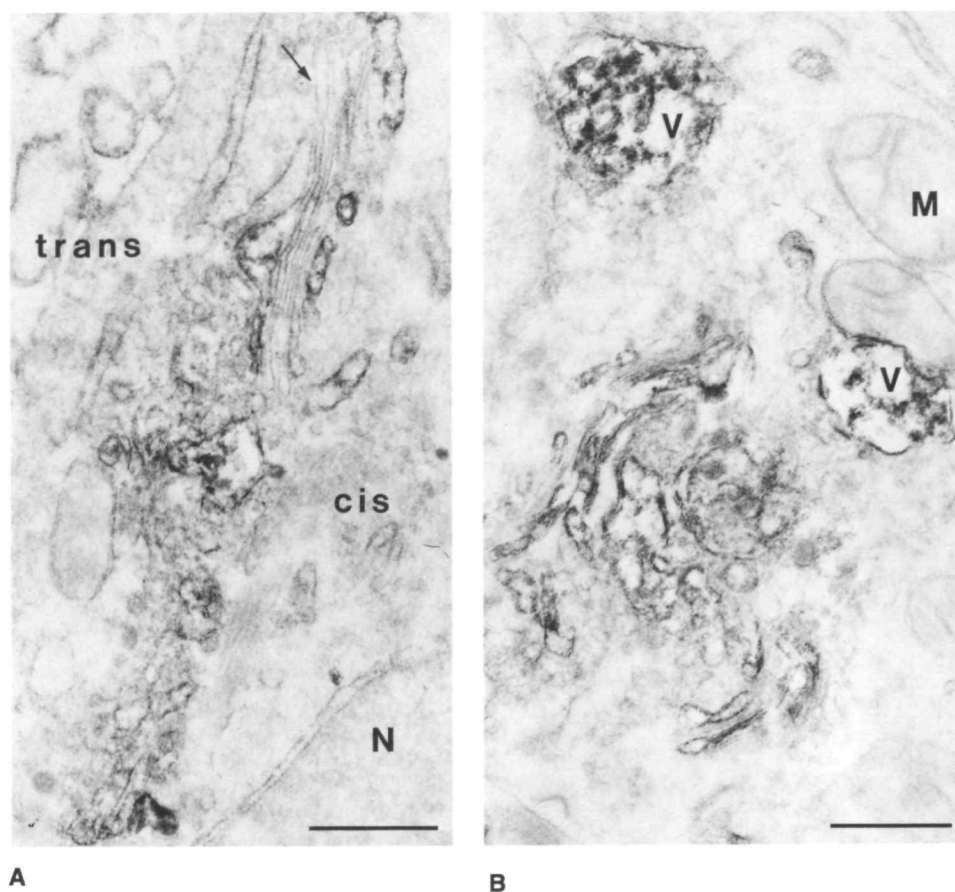


Figure 9. Golgi complexes of HepG2 cells stained for apolipoprotein (apo) A-I. **A.** The reaction products that indicate the sites of apo A-I are present in the Golgi lamellae from cis to trans. However, often the innermost lamellae are weakly stained (arrow). N=nucleus. $\times 44\,800$, Bar=0.5 μm . **B.** On the trans side, large dilated vesicular structures and secretion granules (V) are strongly labeled. M=mitochondrion. $\times 43\,000$, Bar=0.5 μm .

nicity caused by the fixation and embedding routine. The principal group of fixatives used in electron microscopy are the aldehydes, of which formaldehyde and glutaraldehyde are the most commonly used. It has been widely reported that upon glutaraldehyde fixation, ultrastructure is well preserved owing to extensive cross-linking of cellular proteins, but that antigenicity is usually lost.^{41,42} Formaldehyde, on the other hand, does not preserve ultrastructure as well as does glutaraldehyde but has the advantage of allowing retention of antigenicity at least in some proteins, therefore making it the best suited fixative for immunoelectron microscopy.

Investigations on the mechanism of formaldehyde fixation have suggested that primary amines and thiols are the most reactive sites in macromolecules since these sites are subsequently cross-linked to primary amides or guanidyl groups (for reviews see references 43, 44, and 45). The chemical alteration of proteins often leads to a reduction in or a complete abolition of antigen recognition due to the direct alteration of the epitopes or to poor penetration of the antibodies to them after extensive cross-linking. In the present work, we have characterized polyclonal antibodies raised against formaldehyde-modified apo A-I, which were produced in order to circumvent the reduction of antigen recognition induced by the chemical modification of the isolated protein.

Our first step was to investigate the effect of formaldehyde fixation on isolated apo A-I. IEF of fixed compared to unfixed protein revealed a shift of 0.8 to more acidic pIs. This shift is likely due to the reaction of the amino groups present in apo A-I molecules with formaldehyde. Since proteins are rendered more acidic upon blocking of the amino groups by formaldehyde, we were able to demonstrate formaldehyde binding to apo A-I indirectly by isofocusing. The broad zone in the range of pH 4.55 to 4.85, which was visible after focusing of fixed apo A-I, suggests that apo A-I molecules did not react uniformly with formaldehyde. These differences might be due either to varying amounts of formaldehyde being bound to the proteins or to differences in cross-linking to less reactive basic groups.

As a consequence, any antigenic determinant consisting of formaldehyde-changed functional groups would not participate in the immunological reaction, as has already been discussed by several authors.^{44,46} That protein structure can be directly altered by fixation is well established,⁴⁷ and this may adversely affect immunoreactivity.

The reduction of antigenicity upon formaldehyde fixation was demonstrated by ELISA performed with antibodies conventionally raised against untreated apo A-I. Formaldehyde treatment of apo A-I in solution reduced immunoreactivity up to more than 50% of the unfixed

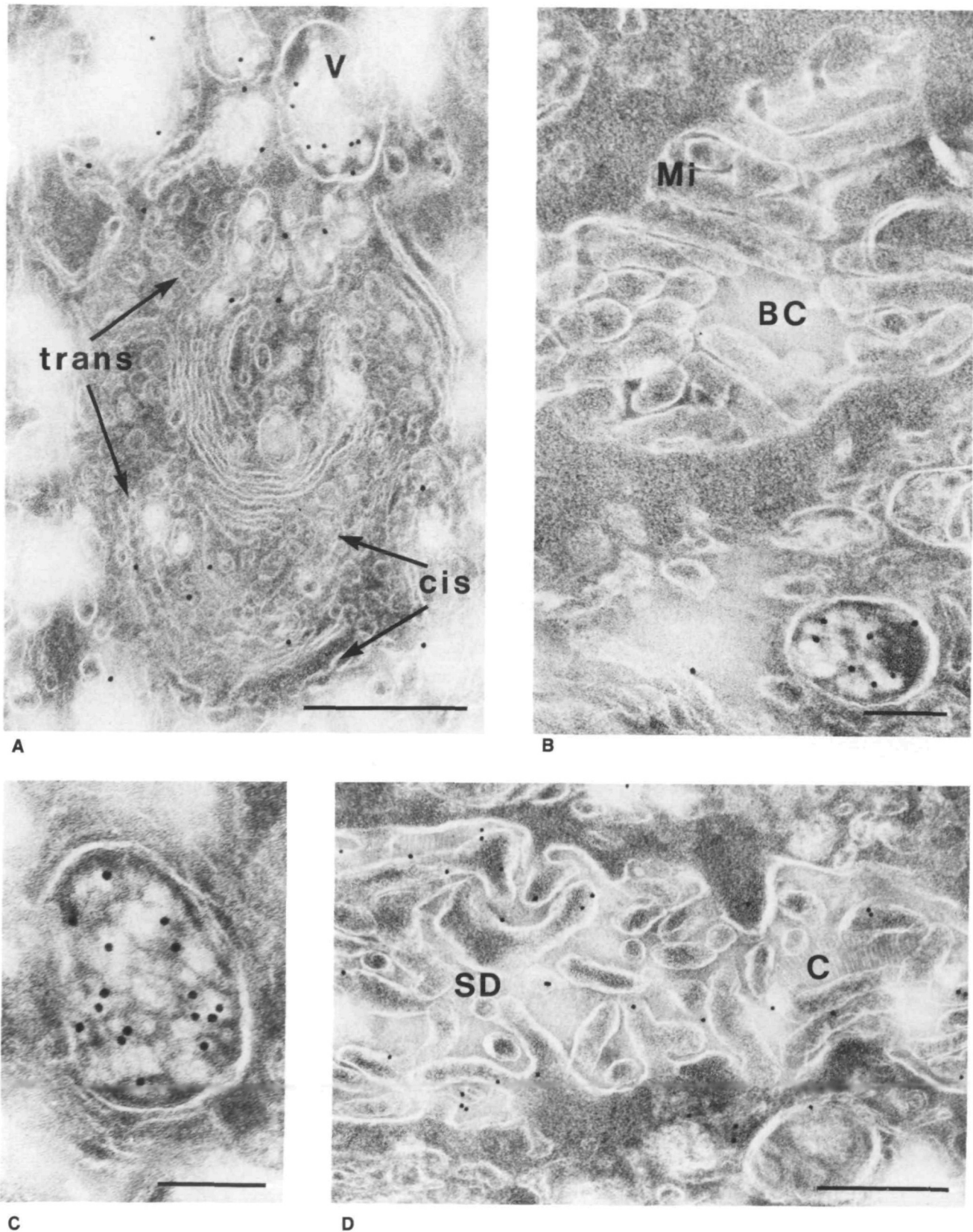


Figure 10. Hepatocytes of a human liver stained for apolipoprotein (apo) A-I by postembedding immunocytochemistry on ultrathin cryosections and using the protein A-gold method. **A.** Vesicular structures and secretion granules (V) at the trans side of a large ring-shaped Golgi apparatus. No gold particles are present on the cisternae and the cis side of the Golgi complex. $\times 60\,500$, Bar = $0.5\,\mu\text{m}$. **B.** Vesicle strongly reactive for apo A-I in close proximity to a bile canaliculus (BC). Mi = microvilli. $\times 75\,600$, Bar = $0.2\,\mu\text{m}$. **C.** High magnification of a vesicle containing gold label indicating the sites of apo A-I. $\times 100\,000$, Bar = $0.2\,\mu\text{m}$. **D.** Immunogold label was consistently present over the space of Disse (SD). C = collagen fibril. $\times 49\,500$, Bar = $0.5\,\mu\text{m}$.

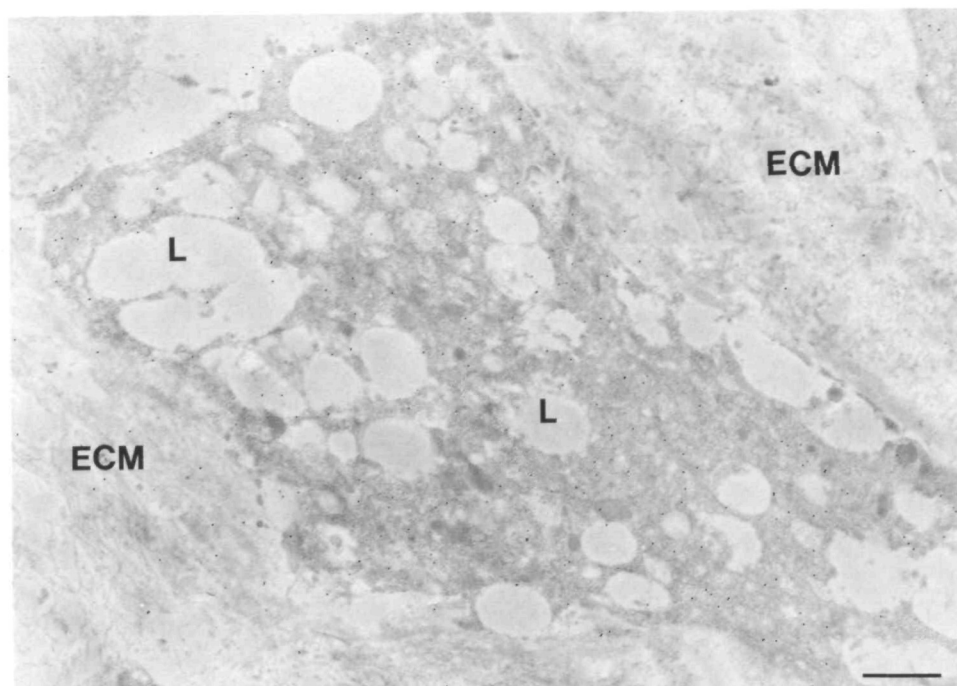


Figure 11. Section of Lowicryl K4M-embedded atherosclerotic plaque from human femoral artery. Postembedding labeling with anti-fix. apolipoprotein (apo) A-I antibodies followed by protein A-gold. The darker staining cell filled with lipid droplets (L) in the main part of the field is part of a foam cell. Note that the cytoplasm of the foam cell is densely labeled, but that lipid droplets are essentially unlabeled. Scattered label is visible over the extracellular matrix (ECM). $\times 14\,000$, Bar = $1\ \mu\text{m}$.

protein. A comparable reduction of immunoreactivity after formaldehyde treatment has been reported for mouse brain spectrin.⁴⁸ The influence of fixation on the intracellular antigen recognition by immunoelectron microscopic techniques was even higher. No specific staining of the antigen could be achieved. The complete loss of immunoreactivity could be explained by further modification of intracellular apo A-I by intermolecular cross-linking to other cellular proteins. In addition, steric hindrance due to extensive cross-linking of all cellular proteins could prevent the antibodies from reaching their antigens.

For these reasons it appeared to be appropriate to try to raise antibodies against apo A-I that had been chemically modified as in the morphological assays. As it is not possible to mimic the complete surrounding of apo A-I in the cell interior, we immunized with the protein chemically modified by formaldehyde in solution. In this way, we aimed to raise antibodies against qualitatively different epitopes: antibodies directed against epitopes that are also present on the unfixed protein and that are not blocked upon fixation, as well as those against antigenic determinants that arose from the formaldehyde treatment.

The ELISA data obtained with our anti-fix. apo A-I antibodies show that there is, indeed, a markedly enhanced recognition of the fixed apoprotein compared to unfixed apoprotein. These data suggest that there are antibodies against determinants specific for formaldehyde-modified apo A-I. The better recognition of the fixed protein was not only gained after affinity chromatography on fixed apo A-I, but was already apparent in ELISA with the unpurified antiserum. The ratio of antigenicity of fixed versus unfixed apo A-I was comparable with antiserum

and affinity-purified antibodies. This shows that affinity chromatography on fixed apo A-I is not the important step for selection of useful subpopulations of antibodies recognizing fixed apo A-I. The results further indicate that there are antigenic sites on apo A-I not affected by formaldehyde fixation, since the antiserum also contains antibodies reacting with the unfixed protein. These results are consistent with those obtained with antibodies raised against unfixed protein, which also recognize a reduced amount of fixed apo A-I.

Immunoreactivity of lipid-bound apo A-I was also increased, as demonstrated with fixed HDL. However, the differences in recognition of fixed compared to unfixed HDL were not as strong as with isolated apo A-I, possibly because of lipid- or protein-covering of fixation-specific sites within the HDL particle. The antibodies reacted only weakly with the other fixed lipoproteins, LDL and VLDL, suggesting that there is no cross-reactivity to apo B, E, or C. It is obvious that the antigenic determinants present on formaldehyde-fixed apo A-I retain lipoprotein specificity. The apoprotein specificity was further verified by immunoblotting of fixed apo HDL. Apart from the pattern of bands that is characteristic of apo A-I, there was slight immunoreaction on one additional band. The potential sources of this faint band may be explored by examining aspects of our preparation procedure. Our lipoproteins were routinely isolated from fresh serum by a well-established, standardized procedure that did not include protease inhibitors. Although the possibility of proteolytic degradation cannot be entirely excluded, a similar band has been documented after *in vitro* plasmin digestion in corresponding preparations of HDL in which protease

inhibitors were included.⁴⁹ Other studies have reported that apo A-I was digested by plasmin at physiological concentrations of each.⁵⁰ In our preparations, the presence of the additional band was not a function of time in storage, but rather was related to the source of the serum, that is, it was present in some donors but not in others. From the point of view of the present study, the crucial aspect was that the band was unrelated to the other apoproteins of HDL, and this was clearly confirmed in our blotting experiments.

Having demonstrated that these antibodies react strongly with fixation-specific determinants on the soluble or lipid-bound protein, we were then able to proceed to immunolocalize apo A-I by morphological methods.

As reported previously, HepG2 cells grown in cell culture synthesize the major human apolipoproteins.^{2,51} Apo A-I is one of the major secretion proteins that accumulates in the medium.^{23,52} Using anti-fix. apo A-I antibodies, it was possible to localize synthesized apo A-I in HepG2 cells by the immunoperoxidase technique, demonstrating that the antibodies react avidly and work in practice when applied to morphological techniques. Reaction products indicating antigenic sites for apo A-I were found in all cellular compartments known to be involved in apo A-I synthesis, since ER, Golgi apparatus, and secretion granules were labeled.

The trans elements of the Golgi complexes were the main compartments stained for apo A-I in both HepG2 cells (as shown by the immunoperoxidase technique) and human hepatocytes (as shown by the protein A-gold technique). These observations correlate with results of a study on chicken hepatocytes,⁵³ which concluded that apo A-I is synthesized in the ER, transported to the Golgi very rapidly, and remains there for a longer period before secretion into the blood.

The preliminary results obtained on an atherosclerotic plaque demonstrate the feasibility of extending our approach to study these pathways in human atherosclerosis.

In conclusion, we have shown that apo A-I recognition in immunoelectron microscopy can be improved by the use of antibodies raised against the protein modified by fixation. This approach will offer new opportunities for the study of the distribution, transport, and cell-protein interactions not only of apo A-I but also of HDL in "normal" cells as well as during the pathogenesis of atherosclerosis.

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Index Terms: apolipoprotein A-I • HepG2 cells • hepatocytes • immunocytochemistry