Monoclonal antibodies to rat C apolipoproteins: production and characterization of a unique antibody whose binding to apoC-I is inhibited by nonionic detergents

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Abstract Four monoclonal antibodies to rat apo (apolipoproteins) C were produced. Three of the monoclonals reacted to apoC-I and one to apoC-III. The IgG monoclonals LRB 21 and LRB 45 recognized a spatially close together or identical apoC-I epitope. The monoclonal LRB 80, however, recognized an epitope that is close to, but distinct from, that recognized by LRB 45 and LRB 21. The antibody LRB 45 recognized an apoC-I epitope that is specific for rat apoC-I, and the antibody did not cross-react with dog or human lipoproteins. Rat apoC-I could be detected in all lipoprotein density fractions, but not in the d > 1.21 g/ml fraction. Freezing and thawing of serum did not alter the antibody antigen binding. However, lipolysis of whole serum resulted in a 30% increase in antigenic epitope expression. Antibody antigen reaction could be inhibited by subcritical micellar concentration of nonionic detergents. The inhibition was specific but could be partially reversed if lipid-containing serum was used as a dilution buffer. On feeding animals a diet of olive oil and cholesterol for 2 weeks, apoC-I levels decreased. — **Wong, L., P. D. Anderson, W. R. Gallaher, and P. S. Roheim.** Monoclonal antibodies to rat C apolipoproteins: production and characterization of a unique antibody whose binding to apoC-I is inhibited by nonionic detergents. *J. Lipid Res.* 1985. **26:** 528–539.

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The C apolipoproteins are a family of apolipoproteins found in all classes of lipoproteins in serum (1). Of all the species of apoC studied, the human apoCs are the best characterized, including their amino acid sequences (1). However, with the exception of apoC-II, the functional properties of the C apoproteins are not clear. There are reports that apoC-I can activate lipoprotein lipase, just like apoC-II (2). There are also reports that apoC-I or its fragments are activators of lecithin:cholesterol acyltransferase (3). The function of apoC-III with respect to lipoprotein metabolism is less clear, although there are reports that apoC-III acts an an inhibitor of lipolysis (4).

In order to examine the structure, function, and metabolism of C apolipoproteins, we have produced monoclonal antibodies to C apolipoproteins. Monoclonal antibodies, with their affinity for a unique and defined epitope on a protein, may be useful in elucidating the structure of lipoproteins. They could define the nature of lipid–protein interaction as well as provide a tool for studying the metabolism of the apolipoproteins. In this study, we wish to report the production and characterization of monoclonal antibodies to rat apoC-I and apoC-III. We also wish to report the characterization of a unique antibody whose binding to apoC-I is inhibited by subcritical micellar concentrations of nonionic detergents.

MATERIALS AND METHODS

Acrylamide, N,N' bis acrylamide, and sodium lauryl sulfate were obtained from BDH (Carle Place, NY). N,N,N',N'-Tetramethylethylene-diamine, X-Omat AR film, and X-ray film holders were from Kodak (Rochester, NY). Sephacryl S-300, DEAE (diethylaminoethyl) Sephacryl, and Sephadex G-50 were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Nitrocellulose paper and filter paper #470 were obtained from Schleicher and Schuell (Keene, NH). Ultrapure guanidine hydrochloride and urea were obtained from Schwarz Mann Inc. (Spring Valley, NY). Culture plates for

Abbreviations: PBS, phosphate-buffered saline; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbant assay; HAT, hypoxanthine, aminopterin, and thymidine medium; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins.

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enzyme immunosorbant assays were from Dynatech Laboratories Inc. (Alexandria, VA). Tissue culture supplies, including DMEM (Delbecco's modified Eagle's media), DPBS (Dulbecco's phosphate-buffered saline), heat-inactivated FCS (fetal calf serum), penicillin, streptomycin, and glutamine were from Grand Island Biological (Grand Island, NY). Tissue culture plates were obtained from Costar Lab Inc. (Cambridge, MA) or from Flow Lab (McLean, VA). Tissue culture flasks were from Corning Glassworks (Corning, NY). ^{125}I was from Amersham (Arlington Heights, IL). Bolton Hunter reagent came from ICN Radiochemical (Irvine, CA). Protein A and all other reagents not specified were from Sigma Chemical Co. (St. Louis, MO). Albino Holtzman rats were from Holtzman Co. (Madison, WI) and Balb/c mice were from Southern Animal Farms (Prattsville, AL). Rabbit antibodies to mouse IgG, IgG2a, IgG2b, IgG3, IgA, and IgM were from Nordic Immunological Labs (El Toro, CA). Peroxidase-labeled rabbit anti-mouse IgG were from Miles Lab. Inc. (Elkhart, IN). PEG4000 (polyethylene glycol) (EM reagent) was from E. Merck (Darmstadt, Germany). P3 cells (P3-X63-Ag8.653 plasmacytoma cell line) were a generous gift from Dr. J. Kearney, University of Alabama Medical Center (Birmingham, AL). Pristane (2,6,10,14 tetramethylpentadecane) was from Aldrich Chemical Co. (Milwaukee, WI). Lipase from *Candida cylindracea* was from Boehringer Mannheim Biochemicals (Indianapolis, IN). MPC11 cells were from the American Type Culture Collection (Rockville, MD).

Immunization and production of hybridomas

It should be noted that the immunization protocol for the production of monoclonals described in the following section resulted in an accidental production of monoclonal antibodies to apoCs. Male Balb/c mice weighing 14–16 g were used for immunization. The mice were injected intraperitoneally and intramuscularly with 50 μg of "apoB" in complete Freund's adjuvant and boosted monthly by intraperitoneal injection of 10 μg of "apoB" in alum. The "apoB" was isolated by passing rat apoVLDL through a 4 M guanidine-HCl Sephacryl S-300 column as previously described (5). The "apoB" peak was judged greater than 90% pure by 10% Tris SDS PAGE (polyacrylamide gel electrophoresis). One month after the primary and booster injections, the mice were prepared for fusion by intravenous injection of 10 μg of "apoB" in PBS 4 days before fusion.

The fusion protocol was carried out according to the method of Fazekas de St. Groth and Scheidegger using P3-X63-Ag8.653 line instead of the Fo cell line described (6). Monoclonality was established by three successive subclonings by limiting dilution, each of which has a cloning efficiency of less than 30%. Ig monoclonals also show discrete bands on gel isoelectricfocusing (7).

Gel electrophoresis

SDS (sodium dodecyl sulfate) gel electrophoresis was performed according to Laemmli (8). A 3–20% linear gradient gel was employed using a Hoefer SE600 series gel apparatus. Two-dimensional isoelectricfocusing SDS-gel electrophoresis was done according to the method of O'Farrell (9). A 5% acrylamide gel containing 6 M urea and 0.1% NP40 (Nonidet P-40) was used for isoelectricfocusing. The 7 M urea basic polyacrylamide gel was as described previously (5).

Immunoblotting

Electrophoretic transfer of proteins from polyacrylamide gel to nitrocellulose paper was performed, without methanol in the buffer, according to Towbin, Staehelin, and Gordon (10).

To quench all the unreacted sites on the nitrocellulose paper, the paper was press-blotted for 16 hr onto a 1% agarose gel containing 0.1% BSA (bovine serum albumin). For incubation with monoclonal antibodies, a 1/5 dilution of media was used. The strips were incubated for 1 hr with shaking with the media diluted in PBS/BSA. After incubation, the strips were washed twice, 10 min each in PBS/BSA with 0.1% NP40 and once with PBS containing 0.05% Tween-20 (PBS-Tween). The strips were then incubated with a 1/1000 dilution of rabbit anti-mouse IgG and washed as described above. ^{125}I-Labeled protein-A (250 ng/ml, approximately 6–8 $\times$ 10^6 cpm/ml) was then incubated with the strip for 1 hr and the strips were washed three times and dried. The strips were then mounted on a piece of paper and radioautographed overnight at -70°C.

Purification of apoproteins

Lipoproteins d $<$ 1.21 g/ml were delipidated, resolubilized, and chromatographed on 2.5 $\times$ 200 cm column as previously described (5) except that Sephacryl S-300 was used instead of Bio-Gel A-1.5m. The final peak, representing C apolipoproteins and apolipoprotein A-II, was collected, dialyzed in 5 mM ammonium bicarbonate, and lyophilized. For separation of C apoproteins, the method of Herbert et al. (11) was used with modifications. A column was constructed using DEAE-Sephacryl and packed to a dimension of 1.5 cm by 25 cm. The column was then equilibrated with 3 L of 0.015 M Tris-HCl, pH 8.2, until the pH of the effluent was stabilized at 8.2. Then the column was equilibrated with 6 M urea, 0.015 M Tris-HCl, pH 8, with 1 mM DTT (starting buffer). About 20 mg of C-A-II apolipoprotein mixture was solubilized in the same urea, Tris-HCl, DTT buffer, filtered through a 0.45 μm filter, and applied to the column. The column was then washed with 50 ml of urea, Tris-HCl DTT buffer.

A 200-ml gradient was constructed using equal volumes of starting buffer and starting buffer with 0.17 M NaCl. Protein was monitored by the dye binding method of Bradford (12).

Iodination

Iodination of protein A was by the chloramine-T method (13). The protein A preparation reacted quite readily with rabbit IgG preparations. There were no reactivities under the same concentration to goat or mouse IgG.

Enzyme-linked immunosorbant assay

ELISA (enzyme-linked immunosorbant assay) was according to Engvall (14) and Tsu and Herzenberg (15), with the recommendation of Rodbard and Weiss (16). Rat lipoproteins of d < 1.21 g/ml were diluted with PBS to a final protein concentration of 10 μg/ml. All coatings used PBS as a diluent. Plates were coated by pipetting 100 μl of the rat d < 1.21 g/ml solution (10 μg/ml) into each well. The plate was then incubated at 25°C for 16 hr. Media were harvested from a cultured hybridoma when cells reached a concentration of 2 × 10^6 cells per ml; the media were diluted to a final concentration of 1/60 with PBS, 1% BSA with 0.1% azide (dilution buffer), mixed with varying dilutions of rat standard serum, and incubated overnight at 25°C. All sample dilutions were made with this dilution buffer. After the incubation, 100 μl of the mixture was pipetted into each well of the coated plate which had been washed previously three times with PBS-Tween. The last drops of wash fluid were removed by throwing the plate face down onto several layers of absorbent material on a table. The mixture was incubated for 1 hr at 25°C in the wells. The plate was again washed. Peroxidase-labeled rabbit anti-mouse IgG was diluted 1000-fold with PBS-1% BSA with no preservatives. Into each well was pipetted 100 μl of the diluted peroxidase-labeled rabbit anti-mouse IgG preparation. The plate was again incubated for 1 hr and washed. Color was developed by the use of 100 μl of ABTS (2,2'-azino-di [3-ethyl benzthiazoline sulfonic acid]) substrate solution (1.8 mM ABTS, 0.003% H_2O_2, 0.1 M phosphate buffer, pH 6.0). The plates were read using a Titertek Multiskan plate reader at 414 nm.

Cotitration

Plates were coated with 100 μl each of 1 μg/ml rat d < 1.21 g/ml lipoproteins overnight at 25°C. The medium used at 1/10 dilution was able to saturate all the apoC-I coated on the plate. Increasing medium concentration to 1/5 did not increase color yield. This observation was interpreted to indicate that the medium used at 1/10 dilution was able to saturate all the apoC-I coated. In a separate experiment, plates were coated with 100 μl of 0.5 μg/ml of rat d < 1.21 g/ml lipoproteins. Similar results were obtained when the data were normalized (17). One hundred microliters of 1/5 dilutions of LRB 21, LRB 45, and LRB 80 were added to separate wells of the coated plates. Mixing experiments, done on the same plate, involved mixing all combinations of one volume each of 1/10 dilution of one medium with another. Also on the same plate was PBS/BSA without antisera. The 1/5 dilution of LRB 21, LRB 45, and LRB 80 served as their own 100% control. The media were incubated in the plates for 1 hr at 25°C, after which the plates were washed three times and incubated with a 1/500 dilution of peroxidase-labeled rabbit anti-mouse IgG for another hour. The plates were again washed three times and color was developed by the use of ABTS substrate solution. To normalize the cotitration, the calculation method employed by Fisher and Brown (17) was employed.

Other analytical procedures

Lipoproteins were isolated as described by Havel, Eder, and Bragdon (18). Protein determination was done according to Lowry et al. (19) as modified by Peterson (20). Protein determination by dye binding was done according to Bradford (12). Amino acid analysis was done on a Glenco amino acid analyzer using the single column method (5). Proteins were hydrolyzed with 6 N HCl in vacuo at 110°C for 22 hr. An internal standard α-amino-β-guanidino-propionic acid-HCl was used. Double immunodiffusion was performed using a 1% agarose gel in 25 mM Tris-Tricine buffer, pH 8.2.

RESULTS

Production of hybridomas

After fusion and HAT selection, there were 30 hybridomas. Of the 30, 6 were found to react strongly with apolipoproteins by the ELISA detection method. We successfully propagated 4 of these 6 hybridomas and subcloned them by limiting dilution. Ig monoclonals also show discrete bands on gel isoelectric focusing (7). Average plating efficiency of clone plates was estimated at 24%. Monoclonality was established by three successive subclonings by limiting dilution. The resulting monoclonal antibodies were characterized by double immunodiffusion with respect to their immunoglobulin class using specific antisera to mouse γ G1, γ G2a, γ G2b, γ G3, γ GM, γ GA, and to light chains λ and κ. All antibodies produced had κ light chains. Two of the four monoclonals produced, LRB 21 and LRB 45, had γ 2b heavy chains, whereas LRB 61 and LRB 80 had μ heavy chains.

Characterization of monoclonal antibody specificity

All antibodies produced reacted to the three lipoprotein classes, i.e., VLDL (d < 1.006 g/ml), LDL (1.006 < d

< 1.063 g/ml), and HDL (1.063 < d < 1.21 g/ml). However, they had no specificity for materials in the d > 1.21 g/ml infranatant fraction. These results suggest that the antibodies produced were against C apolipoproteins (21). This was substantiated by immunoblotting experiments, and the results are summarized in **Fig. 1.** All of the monoclonal antibodies detected fast-migrating peptides, indicative of C apolipoproteins. Three of the antibodies, LRB 21, LRB 45, and LRB 80, seemed to react to the same peptide. LRB 61, however, seemed to react to two peptides. To further characterize the antigenic specificities of the monoclonal antibodies, 2-D isoelectric-focusing SDS-PAGE immunoblotting was carried out. LRB 61 reacted to two peptides having the pIs and electrophoretic mobilities corresponding to that of apoC-III_0 and C-III_3. On the other hand, LRB 21, 45, and 80 did not show any localization indicating that the peptide to which they reacted had a pI greater than 6 or less than 4. The result of the 2-D immunoelectroblotting of LRB 61 is shown in **Fig. 2.**

Since LRB 21, 45, and 80 antigen reactivity fell outside the pH 4–6 range, it seemed likely that they might react to apoC-I. In order to confirm this hypothesis, immunoblotting was carried out on apolipoproteins separated on a 7 M urea slab gel. The results are shown in **Fig. 3.** All three antibodies reacted to a protein having the same electrophoretic mobility as that reported for apoC-I (11). We, therefore, conclude that the three antibodies, LRB 21, 45, and 80, are directed against apoC-I. In comparison, LRB 61 reacted to two fast-migrating peptides, again supporting the conclusion that it was directed against apoC-III_0 and apoC-III_3. Attempts to set up an ELISA using LRB 61 or LRB 80, both IgMs, were not successful. Therefore, there was no further investigation of LRB 61 and LRB 80.

To demonstrate that LRB 21, 45, and 80 did indeed react to apoC-I, rat apoC-I was purified according to the method of Herbert et al. (11). The amino acid composition of the purified protein is compared with that of Herbert et al. in **Table 1.** The purified apoprotein was found to have a pI of 6.41 with a standard deviation of 0.18. When

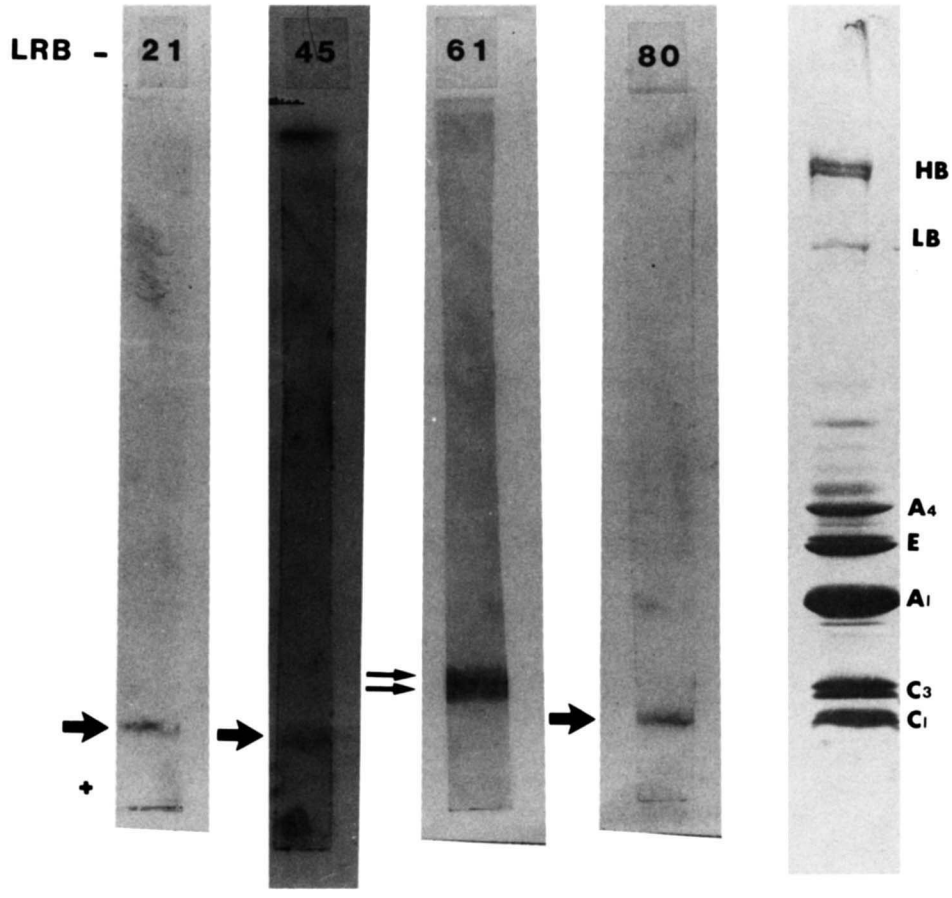

Fig. 1 Radioautogram of Western blot of LRB antibodies to apolipoproteins. A 3–20% gradient SDS gel was run. A Coomassie blue-stained strip is shown on the far right. After gradient SDS-PAGE, the gel was electroblotted, reacted with various monoclonal antibodies, and visualized by radioautography. Arrows indicate area of binding. The results indicate that LRB 21, 45, and 80 reacted to a single species of C apoproteins, whereas LRB 61 reacted to a doublet of the C apoproteins. "HB" and "LB" represent high molecular weight apoB and low molecular weight apoB, respectively, as defined by Sparks and Marsh (22).

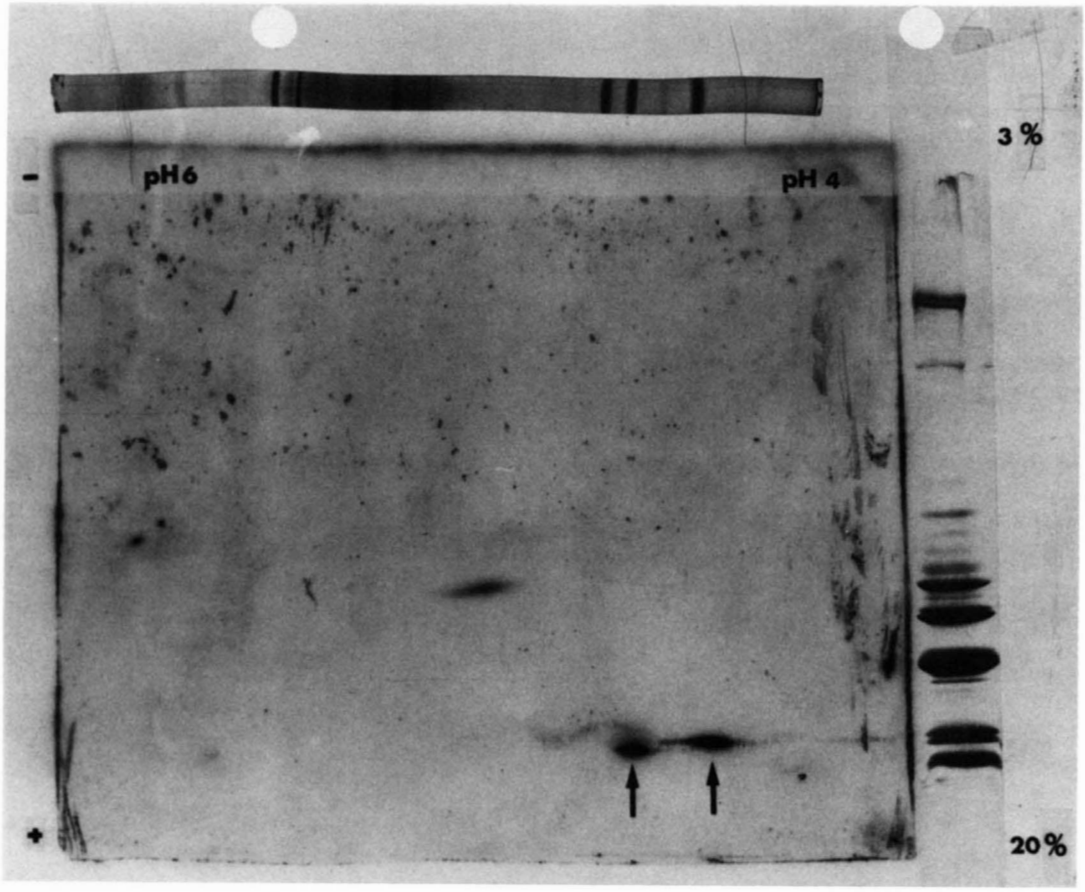

Fig. 2 Radioautogram of a 2-D isoelectricfocusing-SDS gradient PAGE Western blot of LRB 61 with rat d < 1.21 g/ml apolipoproteins. On the top is an isoelectricfocused gel stained with Coomassie blue of rat d < 1.21 g/ml apolipoprotein. On the right is a Coomassie blue-stained strip of d < 1.21 g/ml apolipoprotein as run on 3–20% gradient SDS-PAGE. The two arrows indicate positive reacting spots. The results indicate that LRB 61 reacts to a protein with two different isoelectric points. This is characteristic of apoC-III apoproteins.

this apoprotein was coated to 96-well culture plates at 1 μg/ml, LRB 21, 45, and 80 reacted readily, whereas both monoclonal and polyclonal antibodies to apoE, apoA-IV, apoB, and apoC-III failed to react with this apoprotein. Purified apoproteins A-IV, A-I, E, and apoB coated at 1 μg/ml to plates did not elicit reactivities. Furthermore, the monoclonal antibodies LRB 21 and LRB 45 did not react against human or dog serum over a 10^5-fold dilution range as tested by ELISA, suggesting that the monoclonal antibodies recognized an epitope that is unique to rat apoC-I.

Cotitration of antibodies against apoC-I

In order to determine whether all the antibodies were directed against the same epitope on the antigen, cotitration experiments were carried out (17). The results summarized in **Table 2** indicate that LRB 21 and 45 seemed to recognize the same or spatially close antigenic epitopes (no increase in color development in mixing experiments), whereas LRB 80 appeared to recognize an epitope that is spatially close to, but distinct from, the LRB 21 or 45 (a slight increase, but not a doubling in color development in mixing experiment).

Using standard serum as control, the apoC-I content of a fresh serum sample was determined before the serum was frozen. The serum was then frozen at −70°C and determinations were then carried out on the sample after one, two, and three freeze-thaw cycles over a 6-month period. No effect on the concentration of apoC-I was observed (**Fig. 4**).

Effect of lipase activity on apoC-I determination

The standard serum (labeled as control in Fig. 4) was incubated in the presence or absence of a nonspecific lipase from *Candida cylindracea* (final concentration of 250 U/ml) for 1 hr at 37°C. At the end of incubation, the serum was diluted with dilution buffer and an ELISA was done. The results, summarized in Fig. 4, suggest that hydrolysis increased antigenic epitope expression by 30%.

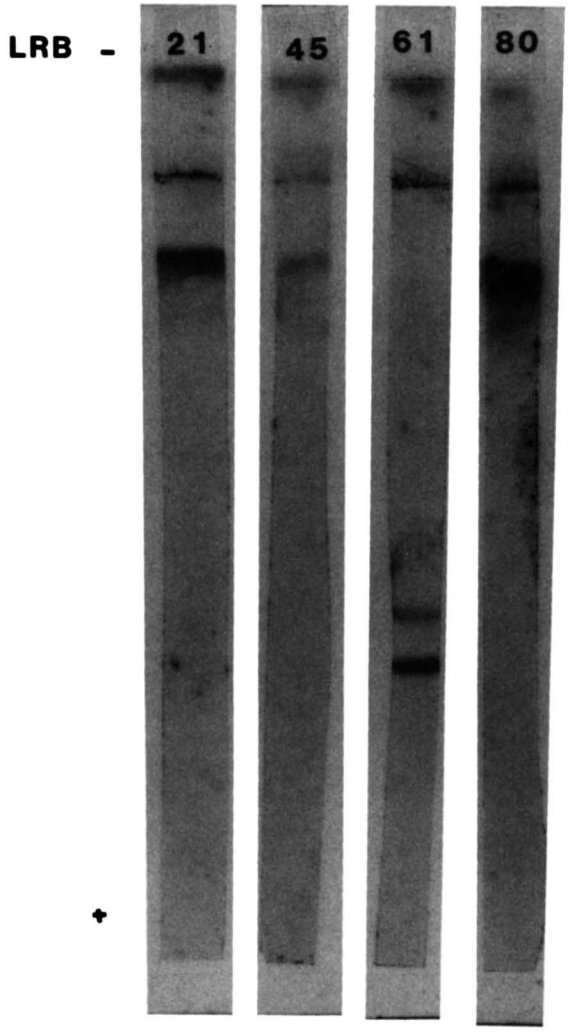

Fig. 3 Radioautogram of 7 M urea basic PAGE Western blot of rat d < 1.21 g/ml apolipoproteins. ApoC-I has a slow mobility in this gel system, whereas apoC-III has high mobility. The combined results of Figs. 1, 2, and 3 are consistent with the hypothesis that LRB 21, 45, and 80 react to apoC-I and LRB 61 reacts to apoC-III.

Determination of apoC-I in rats fed different diets

Three groups of six rats were used for the 2-week diet studies. In the first group, rats were fed ad lib (fed); in the second group, rats were fed ad lib and then fasted overnight (fasted); and in the final group, rats were fed a regular pellet diet supplemented with 2% cholesterol, 20% olive oil (olive oil/cholesterol) for 2 weeks.

The results are summarized in **Table 3.** Upon dietary manipulation, lipid levels in the animals changed. However, the only significant change in apoC-I levels occurred between the olive oil/cholesterol group and the other two groups. These results demonstrate that apoC-I levels in the animal groups can be manipulated by diet.

Effect of nonionic detergents on antibody apoC-I binding

Nonionic and ionic detergents have been used to "normalize" displacement curves in radioimmunoassays of apolipoproteins using polyclonal antibodies. We therefore undertook to determine the effect of nonionic detergents on apoC-I antibody binding. Competitive ELISAs were set up using 1% PBS/BSA dilution buffer with or without 0.05% Tween-20. Rat serum was used as the source for competitive ligands. The results are shown in **Fig. 5A.** On addition of Tween-20 to the BSA dilution buffer, the antibody–antigen reaction was inhibited by 50% (B/B_o = 0.5 or half the total fraction of antibody bound) at low serum concentrations. With increasing serum concentrations, there was an increase in antigen–antibody binding. No difference from control displacement move was observed when detergent was introduced after antigen–antibody binding was allowed to proceed but before introduction of peroxidase-labeled anti-mouse antibody. The points in Fig. 5A were completely superimposable. Furthermore, when pure apoC-I at 1.0 μg/ml was coated onto culture plates and antisera in the presence of PBS/BSA dilution buffer containing 0.05% Tween-20 was added, there was complete inhibition of antigen–antibody

TABLE 1. Amino acid analysis of rat apolipoprotein C-I

Amino Acid	Herbert et al. mol/100 mol	Present Study	mol/mol C-I
Asp	10	10	5
Thr	8	8	4
Ser	6	6	3
Glu	14	14	7
Pro	2	2	1
Gly	2	2	1
Ala	10	10	5
Cys			
Val			
Met	4		
Iso	6	6	3
Leu	10	10	5
Tyr			
Phe	6	12	6
Lys	16	16	8
His	2		
Arg	4	2	1
Trp	1	ND	ND
Total	101	98	49.0
MW	13172.52	13014.6	6507.3

The apoC-I was hydrolyzed under vacuum with 6 M HCl for 22 hr at 110°C. The result, after correction for recovery, is expressed as moles per 100 moles amino acid and moles per mole of apoC-I. The molecular weights were calculated by addition of molecular weights of different amino acids. No correction for glutamine or asparagine was attempted. The result, when compared to that of Herbert et al. (11), was remarkably similar. We note that no methionine was detected in our system. There was more phenylalanine and less arginine than was found by Herbert et al. (11); ND, not determined.

TABLE 2. Co-titration experiments of three antibodies against apoC-I

LRB	21	45	80
21	1.00	0.99 ± 0.01	1.45 ± 0.06
45		1.00	1.49 ± 0.02
80			1.00

The results are averages of two experiments with standard deviations. The numbers have been normalized so that any antibody reacting with itself is set at 1.00. A spatially far away epitope will have a value of 2. This facilitates comparison which indicates that LRB 21 and LRB 45 are reacting against the same or spatially close sites, whereas LRB 80 reacts against a different site from LRB 21 or LRB 45.

reaction. The above observations suggested that detergent interference occurred at the antigen–antibody binding site. To demonstrate that detergent inhibition is not common to all monoclonal antibodies, we repeated the detergent experiment with a monoclonal antibody to rat apoA-I.[2] The result, shown in Fig. 5B, indicated that Tween-20 only partially interferred with the particular antigen–antibody reaction.

The simplest explanation for the observation that increasing rat serum concentrations in the presence of detergent increased antigen–antibody bindings (Fig. 5A) is that the detergent is somehow "captured" by the rat serum. Experiments were carried out to define the detergent capture phenomenon. Specifically, we wished to determine whether serum proteins other than lipoproteins participate in detergent capture. Delipidated apoCs were used as competitors in ELISAs. Three different dilution buffers were used. In the original system, PBS/BSA buffer with or without 0.05% Tween-20 was used as a dilution buffer. In another set of experiments, a 10% dog d > 1.21 g/ml solution, with or without 0.05% Tween-20, was used as a dilution buffer. Finally, a 10% dog serum solution, with or without Tween-20, was used. Dog serum was chosen, since the monoclonal antibody LRB 45 does not recognize dog apoC-I. The results are shown in **Fig. 6.** In the presence of PBS/BSA and Tween-20, apoC-I binding was interfered with. The bound/total (B/B_o) ratio was constant at 0.35. The dog d > 1.21 g/ml fraction gave a much more sensitive displacement curve with the same concentration of apoCs. However, in the presence of Tween-20, there was a complete inhibition of binding. Surprisingly, dog serum in the presence or absence of Tween-20 interfered with binding and no displacement was observed. The results suggest that serum proteins other than lipoproteins do not play a part in detergent capture. The results also suggest that addition of serum to delipidated apoCs can interfere with antibody–antigen binding.

In another set of experiments, rat d < 1.21 g/ml lipoproteins were used as competitors instead of delipidated apoCs. These experiments were intended to demonstrate that detergent capture effect is due to the presence of lipoproteins. With PBS/BSA and Tween-20 as a dilution buffer, the increase in binding with increasing lipoprotein concentration was again observed. With dog d > 1.21 g/ml infranatant as dilution buffer, the same phenomena as PBS/BSA was observed. However, using dog serum as dilution buffer, there was only a shift in the displacement curve. The last set of experiments demonstrated that, with sufficient lipoproteins present, the detergents used may be completely captured and the displacement curve would only be shifted. The results are shown in **Fig. 7.** The combined results of Figs. 6 and 7 suggest that the detergent "capture" effect may be specific to lipoproteins only.

With increasing concentration of Tween-20, there was a progressive decrease in antibody–antigen binding. The results are shown in **Fig. 8.** This detergent effect is not limited to Tween-20. Other nonionic detergents (Triton X-100 at 0.05%, Nonidet P-40 at 0.05%) also showed the same effect. Further, 5 mM sodium decylsulfate as well as 5 mM sodium deoxycholate also inhibited antigen–antibody binding.

DISCUSSION

The monoclonal antibodies produced reacted to all three classes of lipoproteins, i.e., VLDL, LDL, and HDL. However, there was no detectable immunoreactivity over a wide range of dilutions of the d > 1.21 g/ml infranatant. This indicates that apoC-I may be tightly bound to lipoprotein fractions and not easily displaced at high salt concentrations or high centrifugal forces as is the case with apoA-I and apoE (23).

Crumpton and Wilkinson (24), studying the immunochemistry of the sperm whale myoglobin molecule, observed that there was approximately one antigenic epitope per 4,000 to 5,000 daltons of antigen when whole myoglobins were injected into rabbits. If this is correct, intact apoC-I should have at the most two antigenic epitopes. By analyzing the three monoclonal antibodies, we determined that two of the three antibodies were directed toward similar to spatially close epitopes. Furthermore, this epitope seemed to be specific for rat apoC-I only, since no cross-reactivity was detected when human or dog serum was used. One antibody, LRB 80, appeared to be directed toward a different epitope. However, since LRB 80 was an IgM and attempts to set up an ELISA for LRB 80 were not successful, no further investigation using LRB 80 was performed. By using peptic fragments of

[2]Monoclonal anti-rat A-I was generously supplied by Dr. J. J. Thompson, Department of Microbiology and Immunology, Louisiana State University Medical Center.

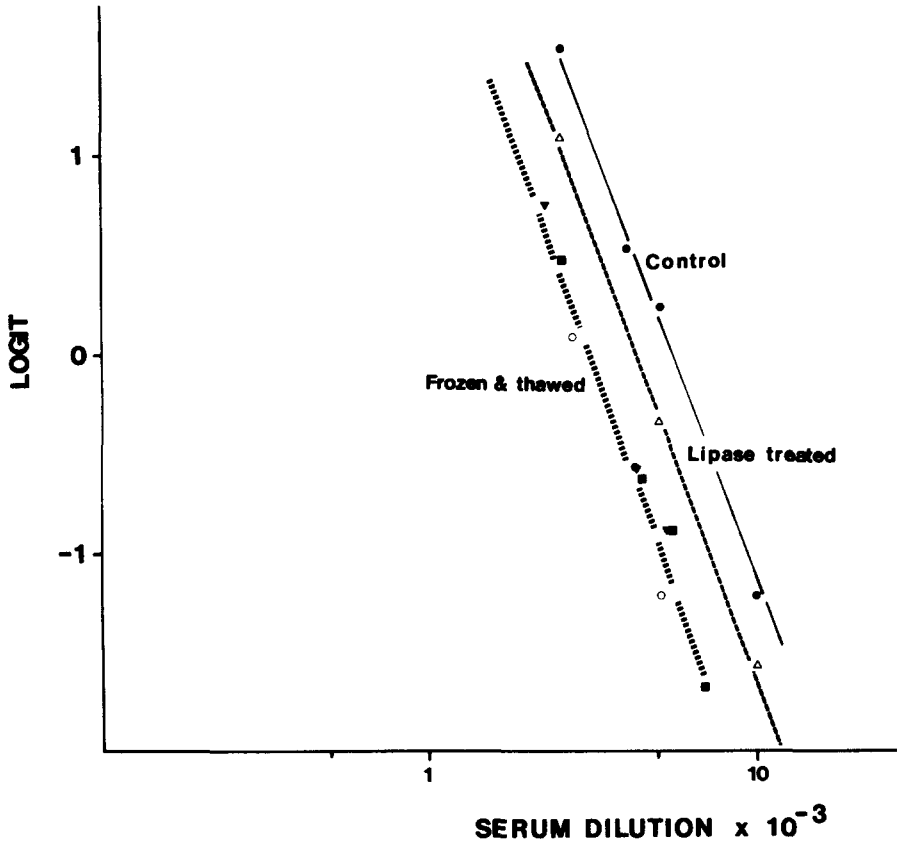

Fig. 4 Competitive assay of a pooled standard serum as compared with fresh serum, and the same fresh serum frozen and thawed three times. Note that freshly bled serum and freezing and thawing cycle experiments are on one line (left line). The right line is the displacement curve of a pooled standard sera, and the middle line is the same pooled standard sera after lipolysis. (■) Represents serum frozen and thawed once; (○) represents serum frozen and thawed three times; (▼) represents fresh serum. Note that some of the points are superimposable on each other. No difference was noted among these three conditions as compared to a pooled standard serum (control), represented by (●). When the pooled standard serum was subjected to lipolysis for 1 hr at 37°C with a nonspecific lipase from *Candida cylindracea* (250 U/ml lipase), there was a 30% increase in antigenic epitope expression (△). Dilutions of serum, frozen, thawed, or after lipolysis were done using dilution buffer PBS/BSA.

TABLE 3. Dietary perturbation can change serum apoC-I levels

Group	ApoC-I	Triglyceride	Total Cholesterol	Cholesteryl Ester	Phospholipid
			mg/dl		
Fed	25.7 ± 9.7	105.7 ± 40.4	75.2 ± 8.1	51.0 ± 6.3	97.7 ± 17.5
Fasted	18.6 ± 4.9	43.1 ± 12.7[c]	58.2 ± 9.8[c]	41.8 ± 8.2[c]	83.7 ± 11.8
Olive oil/cholesterol	13.5 ± 3.9[a,b]	73.0 ± 36.3[a]	112.8 ± 37.7[a,b]	89.7 ± 32.4[a,b]	95.3 ± 5.7[b]

Three groups of six rats each were divided into *a*) fed ad lib, *b*) fasted overnight, and *c*) fed 2 weeks ad lib supplemented with 20% olive oil, 2% cholesterol. The animals were bled between 8 and 10 AM and apoC-I was determined by ELISA. Total cholesterol, triglyceride, free cholesterol, and phospholipid were also determined. Cholesteryl ester values were derived from the difference between total cholesterol and free cholesterol. Serum apoC-I levels were perturbed by dietary manipulation, but not so readily as the serum lipid levels. The values represent mean ± standard deviation of six separate animals.

[a]Significant $P < 0.05$ when olive oil/cholesterol is compared with fed.
[b]Significant $P < 0.05$ when olive oil/cholesterol is compared with fasted.
[c]Significant $P < 0.05$ when fed is compared with fasted.

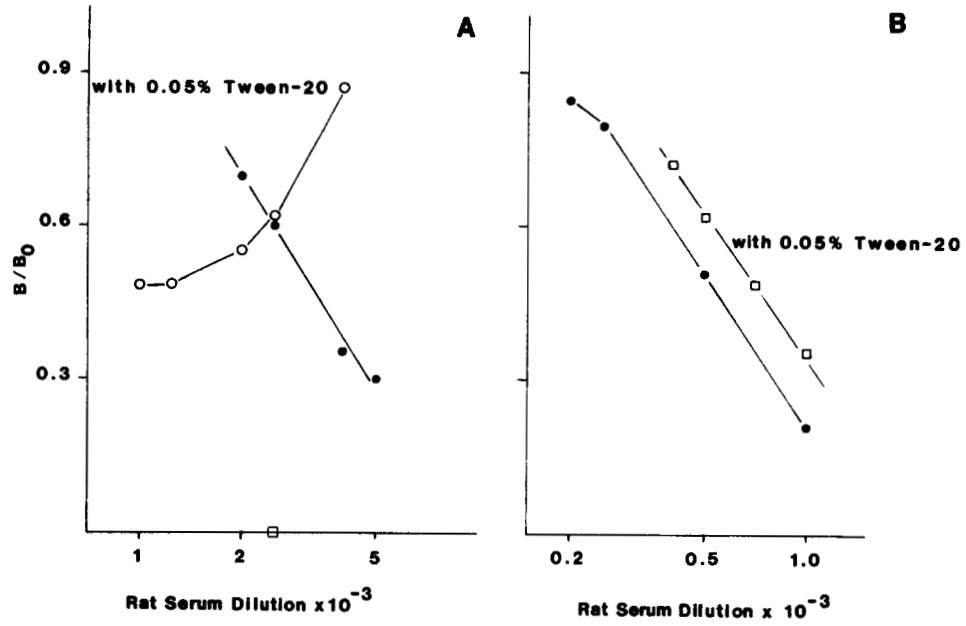

Fig. 5 A: Anomalous behavior of antigen-antibody binding in the presence of subcritical micellar concentration of nonionic detergent. Plates were coated with 100 μl each of rat d < 1.21 g/ml lipoproteins at protein concentrations of 10 μg/ml. Ligand competition is standard pooled rat serum. (○) Represents a standard curve in the presence of 0.05% Tween-20. (●) Represents a normal standard displacement curve in the absence of detergent. When antigen-antibody reaction was allowed to occur before the addition of detergent, the addition of detergent resulted in superimposable displacement curves. When apoC-I at 1 μg/ml was coated onto plates and antibody was added in the presence of detergent, there was complete inhibition of binding (shown by open square). We have determined that in the absence of any competing lipoproteins, Tween-20 completely inhibits antigen-antibody binding. Therefore, one possible explanation for this anomalous observation is that, in the presence of competing lipoproteins, the detergent is "captured" by the lipoproteins and thereby releases the antibody to bind to the immobilized apoC-I. B: Displacement curve of monoclonal antibody to rat apoA-I in the presence or absence of nonionic detergent. Plates were coated with 100 μl each of rat d < 1.21 g/ml lipoprotein at protein concentrations of 0.5 μg/ml. Ligand competition is pooled serum. Not all monoclonal antibody-antigen binding is inhibited by nonionic detergents. In the presence of 0.05% Tween-20, a monoclonal antibody to rat apoA-I is only slightly inhibited. (●) Represents control displacement curve; (□) represents displacement curve in the presence of Tween-20.

apoC-I, we hope to sequence the antigenic epitope of apoC-I for LRB 21 and LRB 45. This, then, would allow us to determine directly whether LRB 80 is directed to the same or different epitope.

Serum from freshly bled rats and a pooled standard serum that had been stored at −70°C for 6 months were compared under different conditions. No difference between the pooled serum and the freshly bled serum was detected whether the freshly bled serum was used fresh, frozen and thawed once, or frozen and thawed three times. Therefore, under conditions where membrane integrity would be destroyed, the apoC-I epitope is not affected. This observation was of practical importance, as it implied that serum samples could be frozen and stored with little effect on the assay. When lipolyzed serum was compared to normal serum, a slight but consistent leftwards shift in the displacement curve was observed (see Fig. 4). This shift accounted for a 30% increase in antigenic epitope expression. These data suggest that a portion of the apoC-I epitope in the normal serum may be buried and may express itself upon lipolysis. Indeed, the proportion of "buried" apoC-I epitope may be higher in animals whose sera are hypercholesterolemic or hypertriglyceridemic. Further investigation of the effect of lipolysis on the degree of exposure of the apoC-I epitope is now underway.

Previously, our group has shown that upon cholesterol feeding of euthyroid rats, there was a significant increase in serum apoB and a decrease in apoE levels, whereas no apparent increase in apoA-I levels was noted (25). We decided to investigate whether the same feeding regimen could affect serum apoC-I levels. As can be seen in Table 3, on feeding rats an olive oil/cholesterol diet, apoC-I levels decreased significantly. The values reported are subjected to the constraints (26) inherent in immunoassays and may, in fact, represent a masking and unmasking of antigenic epitopes. If there is a decrease in apoC-I levels in serum, it suggests that apoE and apoC-I may be removed by the same or similar mechanisms. Alternatively, the decrease in apoC-I level may be attributed to increased serum lipid levels masking the antigenic epitope. This is deemed unlikely, since serum

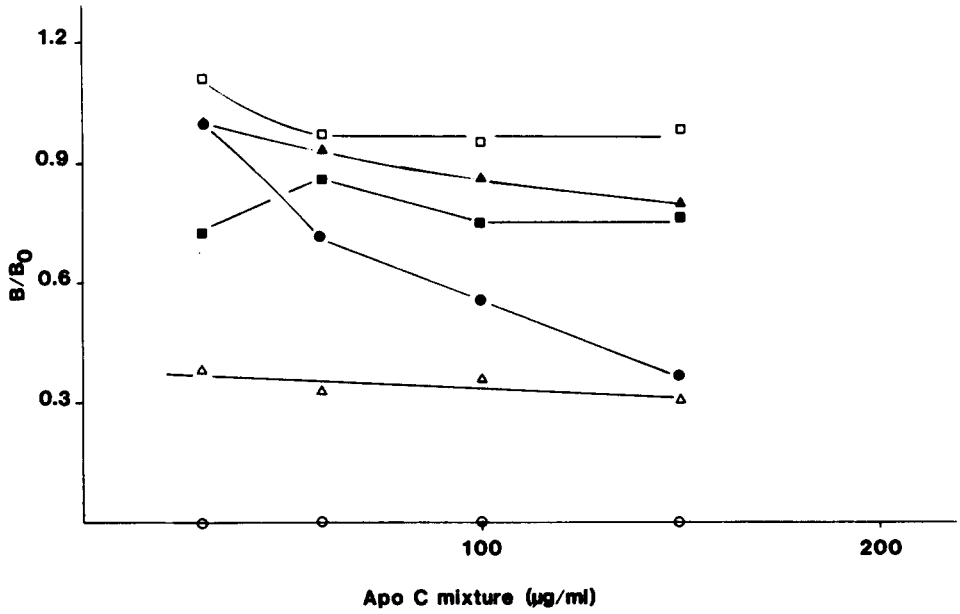

Fig. 6 Competitive ELISA using a mixture of apoCs as competing ligand. Three sets of dilution buffers were used with or without Tween-20. Open symbols represent ELISAs done in the presence of Tween-20. (▲) Represents ELISA done using PBS/BSA as dilution buffer and (△) represents ELISA using PBS/BSA dilution buffer in the presence of 0.05% Tween-20; (●) represents ELISA using 10% dog lipoprotein-free serum as dilution buffer and (○) represents the same ELISA using 10% dog lipoprotein-free serum dilution buffer with 0.05% Tween-20. (■) Represents ELISA using 10% dog serum as dilution buffer and (□) represents ELISA with 10% dog serum dilution buffer and 0.05% Tween-20. The results suggest that lipoprotein-free serum does not have detergent capture effect.

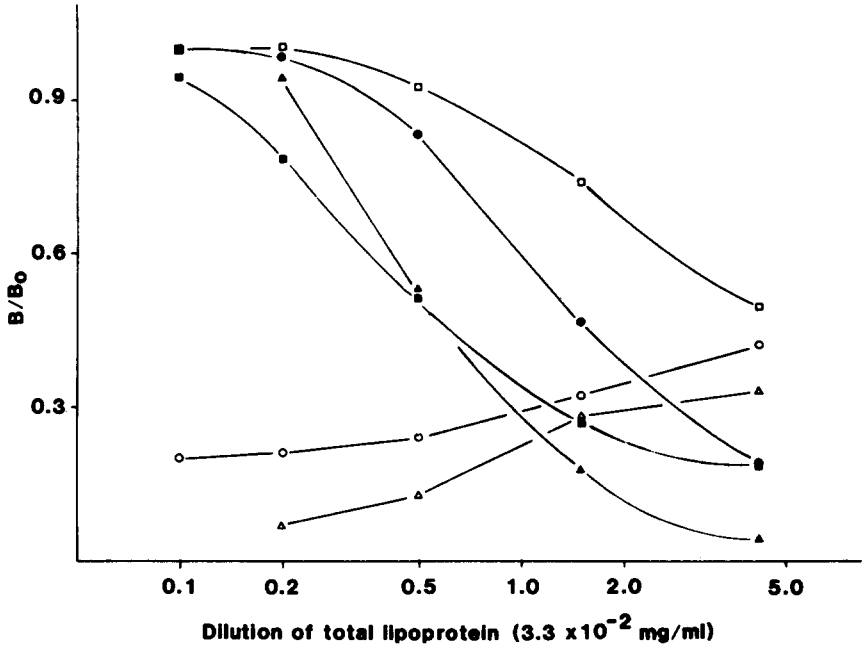

Fig. 7 Competitive ELISA using rat total lipoproteins (d < 1.21 g/ml) as competing ligand. Three sets of dilution buffers were used with or without Tween-20. Open symbols represent ELISAs done in the presence of Tween-20. (●) Represents ELISA using PBS/BSA as dilution buffer and (○) ELISA using PBS/BSA dilution buffer with 0.05% Tween-20. (▲) Represents ELISA using 10% dog lipoprotein-free serum as dilution buffer and (△) ELISA with 10% dog lipoprotein-free serum dilution buffer and 0.05% Tween-20. (■) Represents ELISA using 10% dog serum as dilution buffer and (□) represents ELISA using 10% dog serum and 0.05% Tween-20 as dilution buffer. The results suggest detergent capture is specific to lipoproteins.

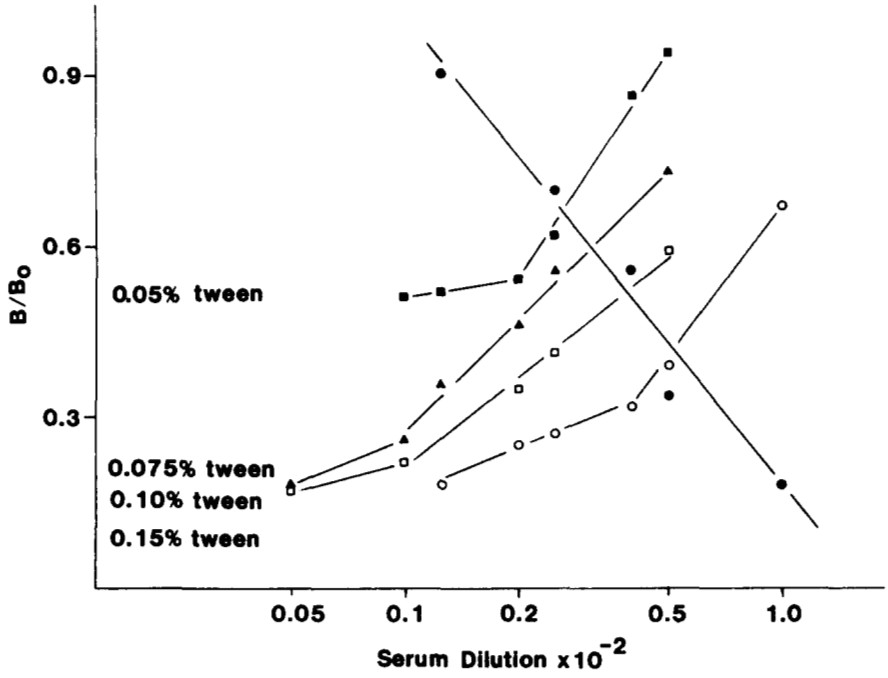

Fig. 8 Degree of inhibition of antibody-antigen interaction is proportional to the amount of nonionic detergents present. (●) Represents a normal displacement curve; (■) represents displacement curve in the presence of 0.05% Tween-20; (▲) displacement curve in the presence of 0.075% Tween-20; (□) displacement curve in the presence of 0.10% Tween-20; and (○) displacement curve in the presence of 0.15% Tween-20.

triglyceride levels actually dropped in the olive oil/cholesterol-fed animals as compared to fed animals. Furthermore, no significant drop in apoC-I was observed between fed and fasted animals, whereas the triglyceride levels were actually halved in fasted animals.

The inhibition of antibody–antigen binding by nonionic detergents is of considerable interest. We planned to use the ELISA for assay of apoC-I in subcellular particles. Detergents were included to break open the subcellular particles before assay. Since most radioimmunoassays of apoproteins in serum employed either ionic (27) or nonionic detergents (28) to "normalize" the standard curve with respect to different lipoprotein fractions, we did not anticipate the results we obtained. In our case, binding was completely inhibited by the presence of the detergent. To demonstrate that this inhibition was not a property of monoclonal antibodies, a different monoclonal antibody was used and binding was shown not to be inhibited. Recently, Watt and Watt (29) and Curtiss and Edgington (7) have shown that antibody–antigen binding of apoB could be inhibited by pre-delipidating LDL with SDS. However, no report to date has shown inhibition of binding by nonionic detergents.

Our data indicate that once the antibody–antigen reaction has occurred, introduction of detergent produced no significant effect. However, we could not determine whether the detergent was acting on the antibody or on the antigen. We have determined that the detergent capture effect is specific to lipoproteins. No other serum protein participated in detergent capture (see Figs. 6 and 7).

Lipoprotein-free dog serum seemed to be a better dilution buffer than PBS/BSA or dog plasma. The result suggests that serum proteins may be important in giving apoC-I an "immunoassayable" conformation. However, using lipoprotein-free dog serum, we could not ameliorate the detergent capture effect that we have seen. Only dog plasma could be used to ameliorate the detergent capture effect (see Fig. 7). The results suggest that only lipoproteins participate in detergent capture. Unfortunately, if dog plasma was used, we could not calibrate primary standard (pure delipidated apoC-I) to secondary standard (pooled serum) since, in the presence of dog serum, there was no displacement by delipidated apoCs (see Fig. 6). Not surprisingly, the detergent capture effect was proportional to the amount of detergent present. With increasing concentrations of detergent, there was a proportional decrease in antigen–antibody binding. It would be of interest to determine whether the detergent binds to a site on apoC-I that occludes antibody antigen binding or whether the detergent changes the conformation of apoC-I thereby making it unrecognizable to the antibody. In conclusion, depending on the antibody produced, use of nonionic detergents to normalize ELISAs and radio-

immunoassays may cause partial inhibition of antibody-antigen binding.

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