Monoclonal antibody to rat apoC: multiple binding to apoC-I on lipoproteins increases its affinity constant

Laurence Wong

Department of Physiology, Louisiana State University Medical Center, 1542 Tulane Avenue, New Orleans, LA 70112

Abstract Using solid phase systems, the kinetics of binding of monoclonal antibody (LRB 45, IgG2b,kappa) to apoC-I and apoC-I on lipoproteins were investigated. At 25°C, the association constant of LRB 45 antibody to apoC-I (3.56×10^6 $M^{-1} \cdot sec^{-1}$) was almost three times slower than the association constant LRB 45 antibody to lipoproteins (10.4×10^6 $M^{-1} \cdot sec^{-1}$). However, the dissociation constant of apoC-I from LRB 45 antibody (0.865×10^{-4} sec^{-1}) was also slower than the dissociation constant of lipoprotein from antibody (1.5×10^{-4} sec^{-1}). Thus, the calculated affinity constant (association constant/dissociation constant) of LRB 45 antibody for apoC-I was approximately half of that for lipoproteins (4.12×10^{10} M^{-1} vs. 6.92×10^{10} M^{-1}). The intrinsic affinity constants for antibody binding to apoC-I and apoC-I on lipoproteins were determined by Scatchard analysis. The intrinsic affinity constant of antibody bound to apoC-I was estimated to be 5.49×10^{10} M^{-1} whereas that of antibody binding to lipoproteins was 30 to 200 times less. Furthermore, ascites fluid from LRB 45 cell lines could immunoprecipitate serum lipoproteins. Thus, it is concluded that there is multiple binding of antibody to apoC-I on lipoproteins. This binding appears to increase the calculated affinity constant (avidity) for antibody-antigen interaction.—**Wong, L.** Monoclonal antibody to rat apoC: multiple binding to apoC-I on lipoproteins increases its affinity constant. *J. Lipid Res.* 1985. **26:** 790–796.

Supplementary key words mononclonal antibodies • kinetics of binding • rat apoC-I • quantification of apolipoproteins

Multivalence of antibody has been recognized for more than 30 years (1). During this period, it has been demonstrated that crosslinking of antibody with antigen, thus forming precipitates, is not essential for immunity. However, during the last 20 years, a number of laboratories have demonstrated through a variety of experiments that effective affinity (avidity, calculated affinity constant) of antibody is significantly enhanced, by virtue of its multivalence, relative to its intrinsic affinity (2, 3). Thus, an IgM with a low intrinsic affinity constant could have a calculated affinity constant 10 to 100 times greater than the intrinsic association constant (3). However, multivalent binding is not only limited to IgM. Due to its divalence, IgG could have a higher calculated affinity constant over its intrinsic affinity (3). In most of the previous studies, estimates had to be made on the effect of different affinities inherent in a population of polyclonal antibodies based on the kinetics measurements that were carried out (3).

With the development of monoclonal antibodies, we have a defined population of antibodies, with a single affinity constant for a single antigenic epitope. With these antibodies, we would not expect immunoprecipitation to occur unless there are repetitive epitopes in the same antigenic molecule. Inasmuch as we were working on an ELISA to rat apoC-I, we wondered whether the multiple binding of monoclonal antibodies to lipoprotein particles would enhance the calculated affinity constant for antibody–antigen binding. In this instance, the multiple binding of antibody to antigen would be due to multiple copies of apoC-I on the same lipoprotein particle. In the present report, we wish to demonstrate that ascites fluid from a monoclonal cell line to rat apoC-I could immunoprecipitate lipoproteins. Moreover, we demonstrate an increased calculated affinity over intrinsic affinity constant due to multiple binding of antibody to lipoproteins.

MATERIALS AND METHODS

Production and characterization of monoclonal antibody LRB 45 has been described in a previous paper (4). The cell line has been subcloned three successive times by the limiting dilution method. The average cloning efficiency was estimated at 24%. The antibody showed

Abbreviations: ELISA, enzyme-linked immunosorbant assay; PBS, phosphate-buffered saline; ABTS, 2,2-azino-di[3-ethyl benzthiazoline sulfonic acid].

distinct multiple bands on gel isoelectric focusing. The antibody is an IgG 2b with kappa light chains and is specific for rat apoC-I. It does not cross react with human or dog apoC-I. Male albino rats were from Holzman Company (Madison, WI). MPC 11 cells were from American Type Culture Collection (Rockville, MD).

Enzyme-linked immunosorbent assay (ELISA)

ELISA was set up as previously described (4). Rat lipoproteins (d < 1.21 g/ml) were diluted with phosphate-buffered saline (10 mM phosphate, pH 7.4, 0.14 M NaCl, 0.1% azide) to a final protein concentration of 10 μg/ml. A flat-bottom plate (Immulon II, Dynatech Lab., Alexandria, VA) was coated with 100 μl of the diluted d < 1.21 g/ml solution. The plate was then incubated at 25°C for 16 hr. Spent media (31.5 μg of mouse IgG 2b) was collected from T-75 culture of LRB 45 cells. The media was diluted to a final concentration of 1/60 with PBS containing 1% bovine serum albumin, 0.1% azide (dilution buffer), and mixed with varying dilutions of rat standard serum. The mixtures were incubated overnight at 25°C. Only one batch of standard serum and one batch of d < 1.21 g/ml lipoproteins were used in these experiments. After the incubation of antibody with standard serum, 100 μl of the mixture was pipetted into each well of the coated plate which had been washed previously three times with PBS containing 0.05% Tween-20 (PBS-Tween). The plate was then incubated for 1 hr at room temperature. After incubation, the plate was again washed three times with PBS-Tween buffer and 100 μl of a 1/1000 dilution of peroxidase-labeled rabbit anti-mouse IgG preparation (Miles Lab., Naperville, IL) diluted in PBS with 1% BSA was added to the wells. The plate was again incubated for 1 hr and washed. Color development occurred by use of 100 μl of ABTS (2,2-azino-di[3-ethyl benzthiazoline sulfonic acid], Sigma, St. Louis, MO) substrate solution (1.8 mM ABTS, 0.003% H_2O_2, 0.1 M phosphate buffer, pH 6.0).

The plate was read using a Titertek Multiskan plate reader (Flow Laboratories, McLean, VA) at 414 nM. Zero standard was determined by doing the ELISA with uncoated wells and 100% standard was simply 1/60 dilution of spent media with no competitor. The results were analyzed by plotting B/Bo versus log serum dilution. B/Bo is defined as amount of antibody bound at various dilutions of serum competitor divided by amount of antibody bound with no competitor.

Scatchard analysis

IgG was partially purified from spent media by 25% (w/v) ammonium sulfate (Aldrich, Milwaukee, WI) precipitation followed by diethylaminoethyl (DEAE) Sephacryl (Pharmacia, Piscataway, NJ) chromatography using 0.1 M phosphate buffer, pH 7.0. Recovery was estimated at 95%. This IgG preparation was adjusted to 31.5 μg/ml of mouse IgG 2b by dilution with PBS-Albumin buffer. Removawell Immulon II plates (Dynatech Lab, Alexandria, VA) were coated with 100 μl of 0.63 μg/ml IgG solution overnight at 25°C. Preliminary experiments with ^{125}I-labeled IgG indicated that approximately 10% of the IgG or 4.2×10^{-14} mol of IgG was bound to the wells. ApoC-I, iodinated with Bolton-Hunter (5) reagent, with a specific activity of 3.01×10^6 cpm/μg was used. Approximately 5×10^4–5×10^5 cpm of apoC-I with or without a 1/500 dilution (7.11×10^{-8} mol/l apoC-I) of pooled standard serum was pipetted to Removawell plate wells. The plate was incubated overnight and then washed ten times with PBS-Tween buffer. The wells were then broken off and counted with an LKB 1274 RIA Gamma counter (LKB Instruments, Rockville, MD).

Kinetics of binding

For determination of rate constant of binding of antibody to apoC-I, Removawell plates were coated with 100 μl per well of an apoC mixture (19.2 μg/ml, purified by passing delipidated d < 1.21 g/ml apoproteins through a 2-meter column with guanidine HCl buffer and collecting bed volume peak [4]) at 25°C overnight. It is estimated that 9.5×10^{-14} mol of apoC-I was coated on the plate. After washing the plate ten times with PBS, 100 μl each of ^{125}I-labeled IgG (3.17×10^{-17} mol/cpm) was pipetted onto the plates. IgG concentration was either 10.51×10^{-10} mol/l or 21.02×10^{-10} mol/l. At specific time points, labeled antibody was removed and the wells were washed ten times with PBS and then counted. Background was determined with uncoated wells at all time points. For determination of the rate constant of binding of antibody to lipoproteins, Removawell plates were coated with 100 μl per well of d < 1.21 g/ml lipoproteins (5 μg/ml). Antibody concentrations were the same as those for apoC-I.

For determination of dissociation rate constants, the plates were coated with the same concentration of apoCs or lipoproteins as above and then 100 μl of labeled antibody (10.51×10^{-10} mol/l) was pipetted into wells and incubated overnight. After incubation, the plates were washed and one set of wells was removed to determine the 100% point. Background was determined as described above with uncoated wells. For the apoC-I dissociation rate constant, 100 μl of apoC mixture (38.4 μg/ml), dissolved in PBS, BSA dilution buffer was pipetted into wells. ApoC-I in the mixture was estimated at 2×10^{-10} mol/ml. At timed intervals, sets of wells were removed and radioactivity was counted. For lipoprotein–antibody dissociation, d < 1.21 g/ml lipoproteins (10 μg/ml) were used as competitor.

Iodination of lipoproteins and apoproteins

Iodination of proteins was by the chloramine-T method. Briefly, to 50 μg of protein in 0.05 M sodium phosphate buffer, pH 7.5, was added 2 mCi of ^{125}I in 20 μl. To

this mixture was added 10 μl of chloramine-T (5 mg/ml in 0.05 M phosphate buffer) and the reagents were mixed for 10 sec. The reaction was stopped by addition of 100 μl of sodium metabisulfite (1.2 mg/ml in 0.05 M phosphate buffer), and finally 0.5 ml of 1% BSA in the same phosphate buffer was added. The mixture was then chromatographed on a Sephadex G50 (Pharmacia, Piscataway, NJ) column pre-equilibrated with 1% BSA phosphate buffer. Iodination efficiency was routinely monitored and found to be between 80–95%. Radiolabeling of apoC-I was by the method of Bolton and Hunter as described (5).

Other analytical procedures

Lipoproteins (d < 1.21 g/ml) were isolated as previously described (4). Protein determination of apoC mixture was by the method of Bradford (6). All other protein determinations were by the procedure of Lowry et al. (7). IgG levels in spent media were determined by using mouse IgG radial immunodiffusion kit (Miles Laboratories, Naperville, IL) after the media had been concentrated 500-fold. The concentration of mouse IgG in solution prepared from ammonium sulfate precipitation of spent media was determined in the same manner.

DATA ANALYSIS

Scatchard analysis

In determining intrinsic affinity constant of apoC-I on lipoproteins, we assumed that labeled apoC-I and lipoproteins are competitors. Under this assumption, we could calculate the reciprocal affinity constant for apoC-I on lipoproteins as:

$$K_{app}^{-1} = K_{apoC\text{-}I}^{-1} \left(1 + \frac{[I]}{K_{lipo}}\right).$$

K_{app}^{-1} is the apparent reciprocal affinity constant, $K_{apoC\text{-}I}^{-1}$ is the apoC-I reciprocal affinity constant, and K_{lipo}^{-1} is the apoC-I on lipoprotein reciprocal affinity constant. [I] is the concentration of apoC-I on lipoprotein added. As we shall see in the Results section, under certain conditions, there is only one population of apoC-I, therefore, K_{app}^{-1} is the reciprocal affinity constant.

Rate constant of association

The antibody–antigen reaction rates were determined using a simplified reaction scheme:

$$A + B \rightarrow X.$$

This is justifiable because antibody–antigen reactions usually have affinity constants in excess of 10^6 M^{-1}, thereby making the reaction essentially irreversible. When antibody concentration greatly exceeds antigen concentration, it could be shown that the antibody–antigen complex X at any given time could be expressed as:

$$X = b\,(1 - e^{-akt})$$

where b represents antigen concentration; a, antibody concentration; and k the association constant. From the equation, it is apparent that the reaction is pseudo-first order where doubling antibody concentration will double the apparent rate of association.

Rate of dissociation

The rate of dissociation is a first order decay reaction. In the presence of an excess of competing antigen, the labeled antibody would be displaced from the antibody–antigen complex. At any given time t, the fraction of antibody–antigen complex B/Bo remaining would be:

$$B/Bo = e^{-mt}$$

where m is the dissociation rate constant, Bo is the amount of antibody bound at time zero, and B is amount of antibody bound at time t after introduction of competing antigen.

RESULTS

The association constant of apoC-I with LRB 45 antibody is illustrated in **Fig. 1.** Using 19.2 μg/ml of an apoC mixture to coat the microtiter plate and 10.51×10^{-10} mol/l of labeled antibody, 50% of the saturable binding occurred in 31.01 min. The association constant was thus

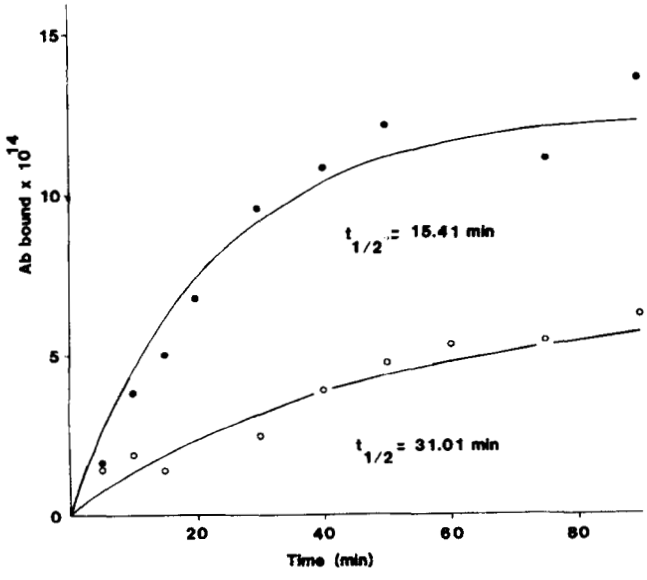

Fig. 1. Rate of binding of anti-apoC-I to apoC-I. (●) Represents experimentally observed values using 21.02×10^{-10} mol/l anti-apoC-I. Lines represent calculated values. (○) Represents observed values using 10.51×10^{-10} mol/l anti-apoC-I. The reaction is pseudo-first order and the observed binding constant is proportional to the actual binding constant and antibody concentration.

calculated to be 3.55 × 10^6 $M^{-1} \cdot sec^{-1}$. These experiments were similar to phage inactivation experiments reported by Hornick and Karush (3). To demonstrate that the reaction is pseudo-first order, the labeled antibody concentration was doubled to 21.02 × 10^{-10} mol/l. As would be expected, the 50% saturable binding time decreased to 15.41 min. From the figure, the calculated apoC-I present on the wells is 9.5 × 10^{-14} mol/l. Similar experiments were done on antibody lipoprotein interactions and the results are summarized in **Table 1.** The averaged association constant for apoC-I with antibody was determined at 3.56 × 10^6 $M^{-1} \cdot sec^{-1}$ whereas that for lipoproteins with antibody was 10.4 × 10^6 $M^{-1} \cdot sec^{-1}$. The calculated apoC-I present on wells in lipoprotein experiments was 1.5 × 10^{-14} mol/l. Therefore, it appeared that antibody binding to lipoproteins was approximately three times faster than antibody binding to apolipoproteins.

The dissociation constant of apoC-I from anti-apoC-I was also determined. Here, labeled antibody (10.51 × 10^{-10} mol/l) was pre-equilibrated with apoC-I precoated on microtiter plates before using an excess (38.4 μg/ml) of apoC to displace the bound labeled antibody from antibody-antigen complexes. The results are shown in **Fig. 2.** Half of the labeled antibody could be dissociated from antibody-antigen complexes in 134 min. Experiments were also conducted on the dissociation constant of antibody bound to lipoproteins. Here the half-time of dissociation was found to be 78 min. Thus, lipoproteins seemed to be better able to displace bound antibody from antibody-lipoprotein complexes than antibody-apoprotein complexes.

Since both the association constant and the dissociation constant of antibody-antigen reactions had been determined, we could calculate the affinity constant of antibody to apoC-I to be 4.12 × 10^{10} M^{-1}. Similarly, the affinity constant of antibody to lipoproteins could be calculated to be 6.92 × 10^{10} M^{-1}. The results are summarized in **Table 2.** By comparing the affinity constants of antibody to apoC-I and lipoproteins, we could determine that there is roughly a twofold difference in favor of antibody lipoprotein binding.

In view of the high association constant for binding of anti-apoC-I to apoC-I, it appeared worthwhile to measure the intrinsic affinity constant of anti-apoC-I to apoC-I.

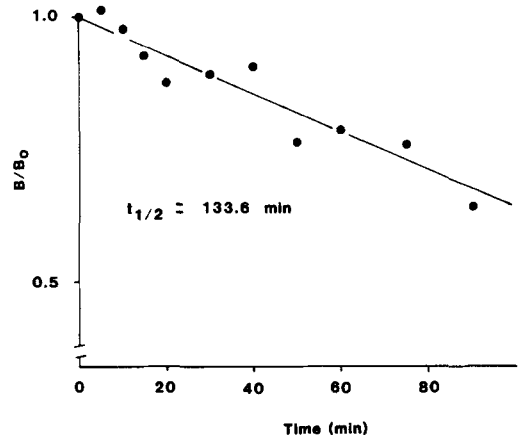

Fig. 2. Rate of dissociation of labeled anti-apoC-I from anti-apoC-I-apoC-I complexes when an excess of apoC-I is introduced. Closed circles represent observed values and the line represents calculated values.

The results of this experiment are shown in **Fig. 3** in the form of a Scatchard plot. This procedure yielded an affinity constant of 5.49 × 10^{10} M^{-1}. It is apparent that the curve is linear; however, it would appear that 1 mol of anti-apoC-I could only bind 1 mol of apoC-I. This result is anticipated due to the random orientation of antibody on the solid support. Measuring an affinity constant for antibody lipoprotein binding was more difficult. This is due to the fact that iodination of lipoproteins will iodinate more than apoC-I; apoB, apoE, apoA-I, and other apoproteins would also have been iodinated. This would make analysis difficult. Therefore, we assume that apoC-I and apoC-I on lipoproteins would act as competitors of each other for binding to antibody. This approach is similar to that reported by Muller (8). If so, we could measure the intrinsic affinity constant of lipoprotein-antibody interaction indirectly by introducing a constant amount (7.11 × 10^{-8} mol/l of apoC-I in the form of serum lipoproteins) of lipoprotein to an antibody-apoC-I system. The results are shown in Fig. 3. From the curve, we could determine that the intrinsic affinity of antibody for lipoprotein was only 3.13 × 10^8 M^{-1}, a 200-fold difference from that of the "observed" affinity constant of 6.92 × 10^{10} M^{-1}. Furthermore, the Scatchard plot extrapolates to 3.86 mol of apoC-I bound per mole of antibody (Fig. 3).

The results above were consistent with the hypothesis of

TABLE 1. Rate of association of anti-apoC-I to pure apoC-I and apoC-I on lipoprotein particles

	Apolipoprotein C-I		ApoC-I on Lipoproteins	
Antibody concentration	10.51 × 10^{10}	21.02 × 10^{10}	10.51 × 10^{10}	21.02 × 10^{10}
$t_{½}$ (min)	31.01	15.41	9.31	6.15
K_{ass} ($M^{-1} \cdot sec^{-1}$)	3.55 × 10^6	3.57 × 10^6	11.8 × 10^6	8.94 × 10^6
Average K_{ass} ($M^{-1} \cdot sec^{-1}$)	3.56 × 10^6		10.4 × 10^6	

The measurements were made at 25°C using labeled anti-apoC-I on microtiter plates. K_{ass} represents the association constant and $t_{½}$ is the half-time of reaction.

TABLE 2. Kinetic and equilibrium parameters for the binding of anti-apoC-I to apoC-I and apoC-I on lipoprotein particles

	K_{ass} ($M^{-1} \cdot sec^{-1}$)	K_{disso} (sec^{-1})	K_{obs} (M^{-1})	K_{intr} (M^{-1})
ApoC-I	3.56×10^{6}	8.65×10^{-5}	4.12×10^{10}	5.49×10^{10}
Lipoprotein	10.4×10^{6}	15.0×10^{-5}	6.92×10^{10}	3.13×10^{8}

The measurements were made at 25°C using labeled anti-apoC-I on microtiter plates. K_{ass} represents the association constant. K_{disso} is the dissociation constant. K_{obs} is the observed affinity constant obtained by dividing K_{ass} by K_{disso}. K_{intr} is the intrinsic affinity constant measured by Scatchard analysis. Note that K_{intr} for apoC-I on lipoproteins assumed that there are two distinct competing populations of apoC-I and C-I on lipoproteins.

multiple binding of antibody to lipoproteins. In an attempt to further define this multiple binding, ascites fluid from an LRB 45 cell line was used in double immunodiffusion experiments to determine whether the antibody was capable of immunoprecipitation of apoC-I from serum and lipoproteins (a predicted consequence of multiple binding). The results are shown in **Fig. 4.** As can be seen, LRB 45 antibody could indeed precipitate lipoproteins. Immunoprecipitation could not be observed for rat lipoprotein-free serum, apoC-I, or apoC-I mixed with rat lipoprotein-free serum. Furthermore, no immunoprecipitation could be observed in fluid phase experiments with labeled apoC-I. These observations are compatible with the hypothesis of multiple binding of antibody to lipoproteins.

To determine whether the observed difference in calculated affinity constant of antibody to apoC-I and antibody to lipoprotein is significant, mixing experiments were carried out using ELISA. The results are summarized in **Fig. 5.** From the figure, it could be seen that competitive ELISA using apoC-I alone as competing ligand and using lipoprotein alone as competing ligand both showed parallel displacement from each other. The concentration of apoC-I in standard serum was estimated at 23.1 ± 0.8 mg/dl. When the displacement curve for lipoprotein was repeated with the addition of 200 ng of apoC-I to each sample, the resultant displacement curve was also parallel to the apoC-I curve.

DISCUSSION

The association constant for antibody binding to apoC-I (3.56×10^{6} $M^{-1} \cdot sec^{-1}$) and antibody binding to lipoprotein (10.4×10^{6} $M^{-1} \cdot sec^{-1}$) was comparable to association constant of trypsin with trypsin inhibitor (2×10^{7} $M^{-1} \cdot sec^{-1}$) (9). The rate is not as fast as that found in dinitrophenol-antidinitrophenol binding (3.7×10^{7} $M^{-1} \cdot sec^{-1}$) (3). The approximate threefold higher association constant with lipoproteins would not, by itself, indicate multiple binding of antibody to lipoproteins.

The dissociation constant measurements were performed with an excess of antigen. This rate constant would be compromised if not enough antigen were used for competition. The estimated apoC-I on microtiter wells that is accessible to antibody binding has been put at 9.5×10^{-14} mol for apoC-I and 1.5×10^{-14} mol for lipoprotein C-I. The amount of antigen used to compete with this binding was estimated to be 10^{-11} mol, a 100-fold excess. This amount of antigen was judged sufficient for competition as the calculated affinity constant (4.12×10^{10} M^{-1}) agreed quite well with that derived from Scatchard analysis (5.49×10^{10} M^{-1}).

In view of the possible errors involved and the sim-

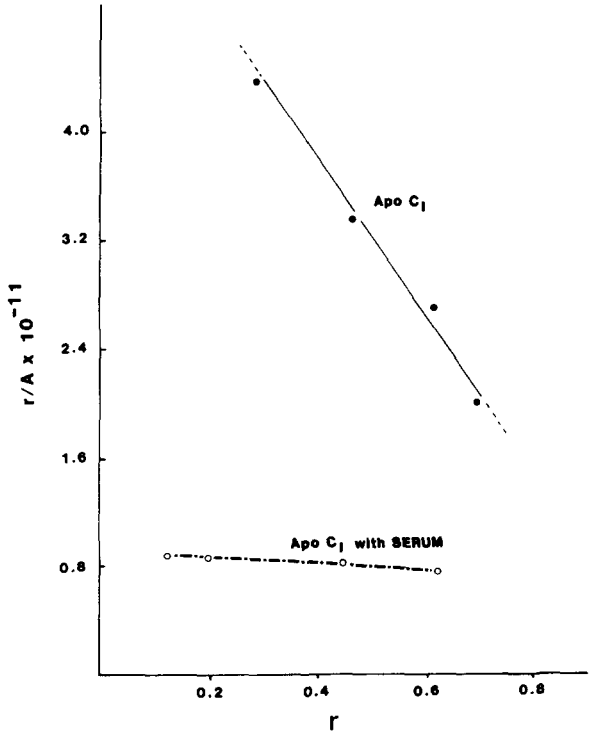

Fig. 3. Scatchard analysis of apoC-I (●) and apoC-I in the presence of 1/500 dilution of pooled standard serum (○). One hundred μl of IgG2 at 0.6 μg/ml was coated onto Removawell Immunlon II plates. Estimated IgG coated was 0.4×10^{-13} mol. ^{125}I-Labeled apoC-I (sp act 2.45×10^{7} cpm/nmol) and apoC-I with 1/500 dilution of serum (7.1×10^{-8} mol/l) was then added to the plate. The plates were incubated overnight at 25°C, washed extensively, and counted; r, defined as moles of antigen bound per moles of immunoglobulin; A, mole of bound antigen.

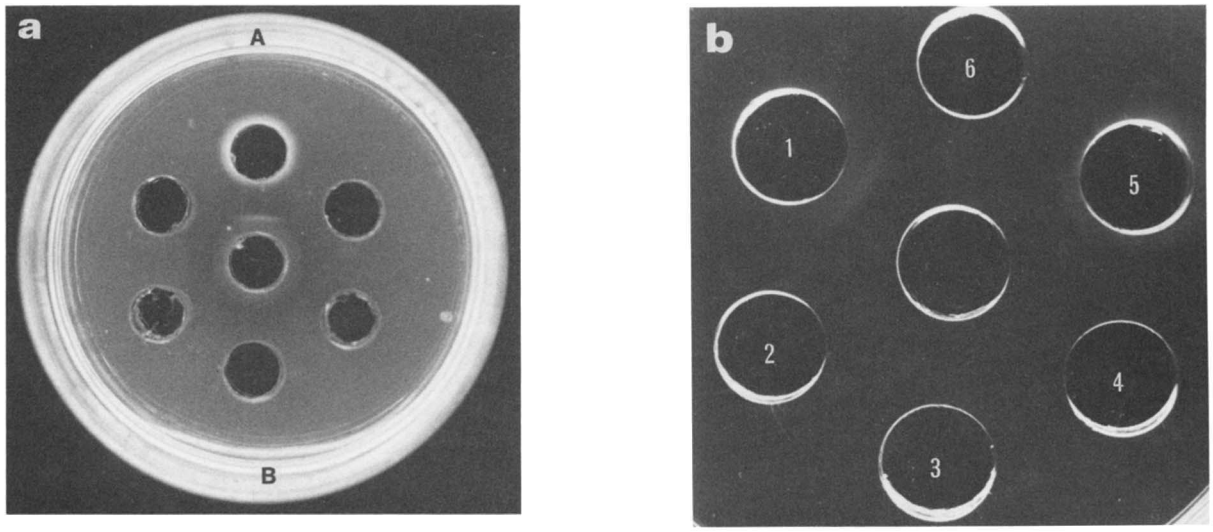

Fig. 4. a, Immunodiffusion of serum lipoproteins by monoclonal antibody to apoC-I. Rat serum, 10 μl, was spotted in the center well of a 1% agarose gel. In well A, 10 μl of ascites fluid from LRB 45 (500 μg/ml) was spotted. In well B, ascites fluid from MPC 11 (γ G2b producer) was spotted. b, Further characterization of ascites immunoprecipitation of serum lipoproteins. Diffusion plates contain 0.8% agarose, 4% PEG 1300–1600 in phosphate buffer. In the center well was spotted 10 μl (125 μg/ml) of gamma globulin fraction of ascites fluid from LRB 45. The other wells are spotted with 10 μl each of 1) serum; 2) rat d > 1.21 g/ml serum; 3) rat d > 1.21 g/ml serum + apoC mixture (143.8 μg/ml); 4) apoC mixture (143.8 μg/ml); 5) 20 μl of rat d < 1.21 g/ml lipoproteins (3.3 mg/ml); 6) phosphate-buffered saline (10 mM phosphate, 0.145 M NaCl, pH 7.4) with 1 g/100 ml bovine serum albumin.

plification that was made in the determination, 4.12 × 10^{10} M^{-1} could indeed be regarded as a reliable affinity constant for antibody to immobilized apoC-I binding. Because of the good agreement between the affinity constants using two differently labeled reagents (apoC-I in one case, antibody in another), the initial assumption that iodination of apoC-I did not change C-I conformation significantly seemed to have been justified. It could not rule out the possibility that labeled apoC-I in solution may form dimers and trimers as other apolipoproteins do (10). This possibility seemed unlikely in view of the observation that the Scatchard plot for apoC-I extrapolated to a value close to 1.0.

The intrinsic affinity constant of antibody binding to lipoproteins was measured indirectly. This was necessary, as currently there is no method that allows ^{125}I-labeling to be specific for apoC-I alone on the lipoprotein particles. Therefore, Scatchard analysis would be jeopardized by low signal to noise ratio caused by other more highly iodinated apolipoproteins on lipoproteins. In the competitive Scatchard analysis, two possibilities were considered: *1*) labeled apoC-I and C-I on lipoproteins existed as two independent populations competing with each other; or *2*) labeled apoC-I was bound to unlabeled lipoprotein particles and that only one population of particles existed.

In the first instance, the intrinsic affinity constant could be calculated at 3.13 × 10^{8} M^{-1} and in the second instance, 2.36 × 10^{9} M^{-1}. In either case, it appeared that the intrinsic affinity constant for antibody binding is 30 to 200 times less than the affinity constant (6.92 × 10^{10} M^{-1}) determined by kinetic measurements. In one experiment, labeled apoC-I was incubated with serum and d < 1.21 g/ml lipoproteins separated by ultracentrifugation. Labeled apoC-I seemed to exist both attached to lipoproteins and in d > 1.21 g/ml infranant in a 1:1 distribution.

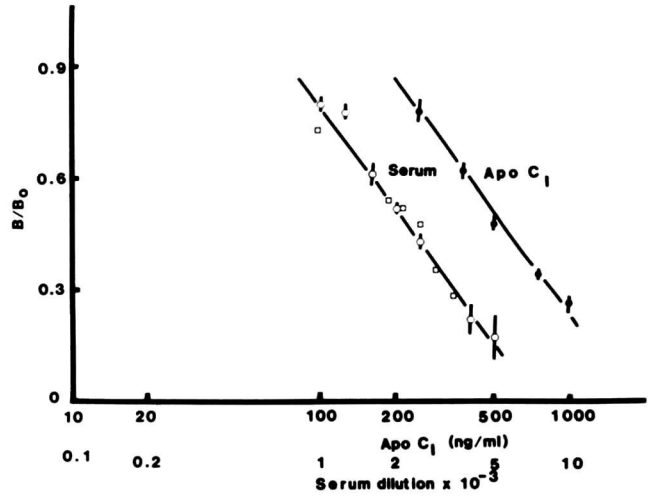

Fig. 5. Determination of affinity constant for anti-apoC-I binding to apoC-I and lipoproteins are similar. Closed circles represent mean (± SD) of four determinations of pure apoC-I by competitive ELISA. Open circles represent standard serum competitive ELISA. Open squares represent standard serum competitive ELISA after 200 ng of pure apoC-I is added to each sample. Note that the serum competition curve and mixing curve are plotted on the dilution scale. In the additional experiment, the abscissa represents the equivalent serum dilution, taking into account the presence of 200 ng of apoC-I.

The difference in affinity constants, determined by kinetics measurements for antibody–lipoprotein binding and antibody apoC-I binding may not be significant. Experiments were carried out by adding 200 ng of apoC-I to standard serum in an ELISA. The results (Fig. 5) suggest that the difference may indeed be insignificant.

The ELISA for apoC-I is not fully validated. In order to fully validate the assay, an independent means of measuring apoC-I must be found. The value obtained by this independent method on a sample must agree with that obtained by the ELISA method. At present, there is no one-step method of quantitatively isolating apoC-I. The assay has been partially validated. On addition of a known concentration of apoC-I to standard serum, the added C-I could be fully recovered by the ELISA method (Fig. 5). In a previous publication (4), it was noted that on lipolyzing serum lipoproteins, there is a 30% increase in apoC-I epitope expression. If this increase in epitope expression is due to "hidden" apoC-I, then the extrapolated value that one antibody molecule could bind to 3.86 molecules of apoC-I on lipoproteins (Fig. 3) must be taken as an underestimation. Consequently, the intrinsic affinity constant calculated from the Scatchard plot would be much lower than reported here. The association constant for apoC-I on lipoproteins would be higher, as would the dissociation constant.

The difference between the affinity constant measured by kinetic experiments and that by Scatchard analysis suggested multiple binding of antibody to lipoproteins. Furthermore, by extrapolating the competitive Scatchard plot (Fig. 3) in the presence of serum competitor, 3.86 mol of apoC-I was bound to one mole of antibody, again suggesting that multiple binding is possible in fluid phase. Since the extrapolated value from the Scatchard plot suggested that there may be an average of more than three apoC-I molecules per lipoprotein molecule, it is likely that antibodies to apoC-I could immunoprecipitate lipoproteins. As shown in Fig. 4, anti-apoC-I could indeed precipitate both serum and d $<$ 1.21 gm/ml lipoproteins. No precipitation was observed with lipoprotein free serum. Moreover, using ^{125}I-labeled apoC-I, we were not able to determine apoC-I anti-apoC-I precipitation in fluid phase.

In conclusion, we have demonstrated that multiple binding of a monoclonal antibody to lipoproteins increased the affinity constant for binding. Furthermore, this multiple binding is capable of immunoprecipitation.

I wish to thank Dr. L. Byers, Department of Chemistry, Tulane University, for his comments and suggestions. The encouragement and support of Dr. P. S. Roheim is gratefully acknowledged. The skillful technical assistance of P. D. Anderson is greatly appreciated. I thank Alfreda Davis and Lawrie Lehmann for the skillful secretarial preparation of this manuscript. This work was supported by NIH program project grant #HL 25596 to Paul S. Roheim and by grant AHA 83-1028 from the American Heart Association, with funds contributed in part by AHA Louisiana Affiliate, to L. Wong.

Manuscript received 19 March 1984.

REFERENCES

1. Eisen, H. N., and F. Karush. 1949. Significance of valence in antibody interactions. *J. Allergy.* **20:** 393–399.
2. Sarvas, H., and O. Makela. 1970. Haptenated bacteriophage in the assay of antibody quantity and affinity: maturation of an immune response. *Immunochemistry.* **7:** 933–943.
3. Hornick, C. L., and F. Karush. 1972. Antibody affinity. II. The role of multivalence. *Immunochemistry.* **9:** 325–340.
4. Wong, L., P. D. Anderson, W. R. Gallaher, and P. S. Roheim. 1985. 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.* **26:** 528–539.
5. Bolton, A. E., and W. M. Hunter. 1973. The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. *Biochem. J.* **133:** 248–254.
6. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **72:** 248–254.
7. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
8. Muller, R. 1980. Calculation of average antibody affinity in anti-hapten sera from data obtained by competitive radioimmunoassay. *J. Immunol. Methods.* **34:** 345–352.
9. Green, N. M. 1957. Kinetics of the reaction between trypsin and the pancreatic trypsin inhibitor. *Biochem. J.* **66:** 407–415.
10. Barbeau, D. L., and A. M. Scanu. 1976. Ultracentrifugal behavior of apolipoprotein A-I of rhesus monkey (*Macaca mulatta*). *J. Biol. Chem.* **251:** 7437–7441.