Studying Low-Density Lipoprotein−Monoclonal Antibody Complexes Using Dynamic Laser Light Scattering and Analytical Ultracentrifugation†

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ABSTRACT: Monoclonal antibody complexes have proven very useful in the study of low-density lipoproteins (LDLs). Thus, complexes composed of two different monoclonal antibodies, selected from a panel of 11 different antibodies, and LDL have been employed to map apolipoprotein B (apoB) on the surface of the LDL. In this way, apoB was found to surround the LDL as a ribbon with a bow [Chatterton, J. E., et al. (1995) *J. Lipid Res. 36*, 2027−2037]. Moreover, monoclonal MB19, which recognizes a polymorphic site, has been employed to quantitate the two different allelic forms of apoB found on LDL in human sera, and in this way, we assessed the effect of most of the known common polymorphisms of this protein as well as detected the depletion of the normal allele product in two forms of familial defective apoB-100 [Chatterton, J. E., et al. (1995) *Biochemistry 34*, 9571−9580; Pullinger, C. R., et al. (1995) *J. Clin. Invest. 95*, 1225−1234]. In this paper, these studies have been extended by examining by dynamic light scattering and sedimentation velocity techniques the complexes formed with only one antibody, and complexes formed using two antibodies. Our data show that the largest complex formed with a single monoclonal antibody was that of an LDL dimer; no larger, nonspecific complexes were present. With two antibodies, a variety of complexes were seen. Thus, monoclonal antibodies MB47 and 4G3, which bound about 55° apart, formed a very stable dimer. Monoclonal antibodies MB47 and 2D8, which bound 136° apart, formed a very stable tetramer, with four LDLs held together in probably a circular structure with four monoclonal antibodies. Finally, monoclonal antibodies 2D8 and 1D1, which bound 86° apart, probably formed a less stable LDL tetramer, held together by three to four monoclonal antibodies. A rationale for these structures is discussed, as well as the biological relevance of these complexes.

Low-density lipoproteins (LDLs)[1] are associated with a single, large 4536-amino acid glycoprotein, apolipoprotein B-100 (apoB-100) (*1*). ApoB-100 is the ligand for the LDL receptor, which clears LDL from the plasma (2). It has been shown that elevated levels of LDL in the blood are correlated with increased risk of atherosclerosis and heart disease. ApoB-100 is in fact a very important determinant in causing elevated levels of LDL as seen in the disease familial defective apoB-100, in which a single mutation in apoB diminishes the level of binding to the LDL receptor and causes increased levels of LDL in the blood (*3*). LDLs are spherical particles consisting of a core of cholesterol esters together with some triglyceride, surrounded by a monolayer of phospholipid and unesterified cholesterol in which a single copy of apoB is embedded.

Our group has clearly defined the configuration of apoB on LDL using pairs of monoclonal antibodies and an electron microscope (*4*, *5*). This was possible because there is only a single copy of the apoB protein on the LDL. Adding two different monoclonal antibodies to LDL yielded an angle between the binding sites. By examination of a large number of LDLs, it was possible to identify 50−100 angles for each antibody pair, and to plot a histogram from which the average angle could be determined. When all the information was collected and analyzed, apoB was found to surround the LDL like a ribbon, ending with a bow.

One troubling feature of these studies, however, was why so few antibodies could be seen binding to the LDL. For example, panels a and b of Figure 1 of ref *4* and panels a and b of Figure 3 of ref *5* were selected because they clearly showed pairs of antibodies binding to the same LDL; nevertheless, most of the LDL in these micrographs had no visibly bound antibodies. Was this deficit due to the antibodies being hidden when bound either above or beneath the LDL, as we speculated? Or could most of the antibody have been dissociated from the LDL, even though the grids were prepared within about 90 s after dilution of the solutions by 10-fold? Or could our preparations be proteolytically damaged so that we saw only a minority of the LDL which displayed each particular epitope?

Conversely, if all these LDLs actually had antibodies bound, what about aggregation? Why did we never see

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[1] Abbreviations: LDL, low-density lipoprotein; apoB, apolipoprotein B100; Ab, antibody; mAB, monoclonal antibody; S, Svedberg; F, Ficks.

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precipitates, or only rarely aggregates even the size of tetramers?

It was questions such as these which led us to the experiments described in this paper. Once we started, however, this fascinating study led to the experiments shown below. Sometimes essentially all of the LDL and bivalent monoclonals formed LDL dimer, and often with the addition of two monoclonal antibodies, all of the LDLs would form complexes larger than an LDL dimer. Using dynamic laser light scattering and analytical ultracentrifugation, we studied how different ratios of monoclonal antibodies affected the sizes of these complexes. At equivalence, complexes of maximum size were expected. In previous dynamic light scattering studies, we showed (*6*, *7*) that the addition of one bivalent monoclonal antibody may form an LDL dimer, a technique used to assess the ratio of the two allelic forms of LDL in normal persons and those with familial defective apoB-100. Two monoclonal antibodies spaced about 90° apart could also form a cyclic dimer. If we spaced our monoclonal antibodies more than 120° apart, we would expect a longer "daisy" chain with a larger number of LDLs in the complex. Figure 1 shows the various complexes expected to form upon the addition of one or two monoclonal antibodies.

Dynamic laser light scattering provided information about the diffusion coefficient of the complexes formed. To determine the number of LDLs in each complex, we needed to calculate the average molecular mass of the complexes. Molecular masses required both the diffusion coefficient from the dynamic laser light scattering and the sedimentation coefficient from the analytical ultracentrifugation. Here we present our data using differently spaced monoclonal antibodies on apoB, and the effects they have on the sizes of the LDL−antibody complexes that formed.

MATERIALS AND METHODS

LDL Isolation. Total LDL (isolated from a density cut from 1.019 to 1.055 g/mL) for light scattering and analytical ultracentrifugation was obtained from human plasma by the sequential flotation procedure described previously (*4*). Briefly, for most subjects, sufficient LDL was obtained from 30 mL of blood. Protease inhibitors were added immediately after the blood was drawn, and their final concentrations were 24 μg/mL Polybrene, 2 mM benzamidine, 5 mM ϵ-aminocaproic acid, and 20 μg/mL soybean trypsin inhibitor. In addition to 0.04% EDTA (w/v), we added 0.05% sodium azide (w/v) and 0.005% gentamycin sulfate (w/v), and a constant background concentration of 40 μM ascorbic acid was maintained throughout the isolation to protect LDL from oxidation (*8*). The first centrifugation at a density of 1.063 g/mL was performed at 44 000 rpm for 22 h at 20 °C in a Beckman Ti-70.1 rotor and a Beckman L5-65 ultracentrifuge (Beckman Instruments, Palo Alto, CA). This centrifugation resulted in VLDL, IDL, and LDL floating to the top, and these lipoproteins were removed and subjected to a second centrifugation at a density of 1.021 g/mL at 20 °C. The bottom layer (LDL), from the second centrifugation, was adjusted to a density of 1.055 g/mL (20 °C), and the centrifugation was repeated a third time to yield total LDL which was isolated from the top of the centrifuge bottle.

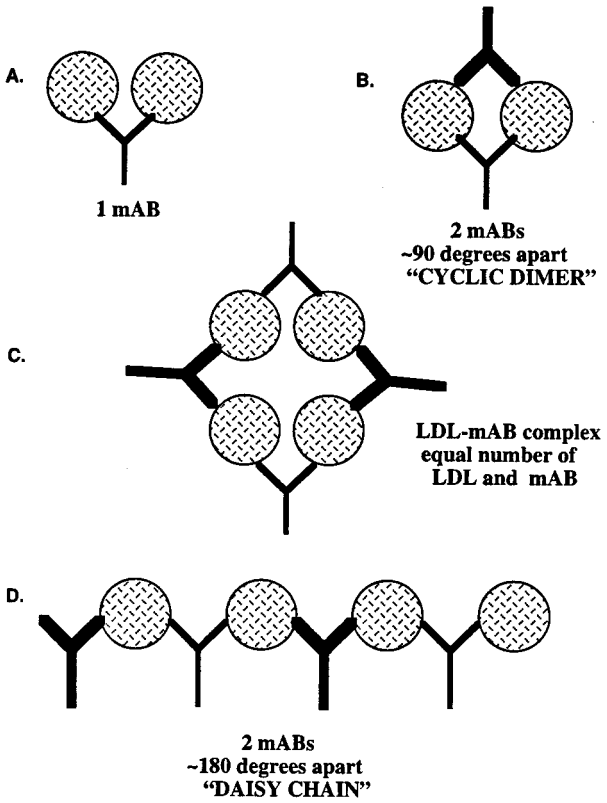

FIGURE 1: Various LDL−monoclonal antibody complexes that can be formed between bivalent, monoclonal anti-apoB antibodies and LDL which contain a single apoB. (A) The simplest complex formed is that of an LDL dimer, two LDLs bound by one monoclonal antibody. (B) A "cyclic dimer", which also contains two LDLs held together by two monoclonal antibodies, can be formed using two monoclonal antibodies that have epitope sites 90° apart. (C) An LDL−antibody closed-ring complex containing equal numbers of both LDL and antibodies can also be formed (center) and is illustrated using a complex that contains four LDLs and four monoclonal antibodies. (D) A "daisy chain" complex that contains indefinite numbers of both LDLs and antibodies that would be generated by antibodies with epitope sites about 180° apart. LDLs are depicted as circular particles, while one type of antibody is drawn as a thin Y-shaped figure and the other antibody as a thick Y-shaped figure.

Total LDL was placed on a Pharmacia PD-10 column packed with Sephadex G25M that had been equilibrated with a 10% BSA solution and thoroughly washed, and the LDL was eluted with Mock [0.195 M NaCl, 0.04% EDTA (w/v), and 0.05% sodium azide (w/v) (pH 7.6)], leaving out the ascorbic acid which interfered with the Lowry protein quantitation measurements. LDL used for the ultracentrifugation experiments was also eluted as mentioned previously, but with Mock buffer that was lacking sodium azide due to its interference with light absorption which was used to follow the migrating boundaries during sedimentation velocity analysis. Any large particulates were removed by centrifugation in a microfuge for 1 h at 12K rpm and 4 °C. The supernatant solution was stored in a 1.5 mL microfuge tube under nitrogen at 4 °C. The protein concentration in the supernatant solution was determined by a modified Lowry assay (*9*) using bovine serum albumin (RIA grade, Sigma, St. Louis, MO) as a standard, corrected for apoB hyperchromicity (*10*). The molar LDL concentration, which is numerically equal to the molar apoB concentration, was determined by dividing the protein concentration by the molecular mass of the apoB protein (513 000 g/mol) (*11*).

Monoclonal Antibodies. Monoclonal antibodies MB47, 2D8, 4G3, and 1D1 are mouse monoclonal antibodies raised against human LDL. The production, isolation, and characterization of these antibodies have been previously described for MB47 (*12*−*14*) and for monoclonals 1D1, 2D8, and 4G3 (*15*−*17*). The production and purification of these antibodies will be discussed below.

Antibody Purification. The IgG subclass containing the anti-LDL antibody was isolated from the ascitic fluid of hybridoma-bearing mice by elution from a column of protein A coupled to Sepharose 4B (Pharmacia LKB, Piscataway, NJ) or from a human LDL-Sepharose 4B column (*18*). The antibody was eluted from the LDL column with 0.1 M citric acid or 3 M potassium iodide or was eluted from the protein A column with 0.1 M acetic acid or a stepwise pH gradient (*19*). 1D1 and 2D8 were identified as IgG1 immunoglobulins, while MB47 and 4G3 were identified as IgG2a immunoglobulins.

A typical ascities purification yielded 3−9 mg/mL antibody. Because the yields were high, the antibodies were diluted to 0.2−0.3 mg/mL with TBS-azide [0.15 M NaCl, 10 mM Tris, and 0.05% NaN_3 (pH 7.4)]. Antibody dilutions were centrifuged at 12K rpm and 4 °C for 1 h to remove large particulates and then aliquoted into eight 0.4 mL aliquots. The protein concentration was estimated spectrophotometrically at 280 nm, using an extinction coefficient of 1.36 mL mg^{-1} cm^{-1}, while the molar concentration was calculated using a molecular mass of 150 000 g/mol for each antibody. Again, because sodium azide interferes with light absorption that was used to follow migrating boundaries in the sedimentation experiments, the purified antibodies were then placed on a G50 Penefsky column (*20*) to remove NaN_3 and eluded with 0.15 M NaCl and 10 mM Tris (pH 7.4).

Dynamic Light Scattering. LDL and LDL−antibody complex diameters (from the diffusion coefficient calculated as a Stokes radius) were determined with dynamic light scattering using a NICOMP model 270 particle sizer (Particle Sizing Systems, Santa Barbara, CA) equipped with a 2017 argon ion 2 W laser (Spectra Physics Lasers, San Francisco, CA) and at a wavelength of 514.5 nm. This instrument uses the autocorrelation function to calculate the diffusion coefficient of the macromolecule from the fluctuations in the scattering intensity. The Stokes radius was then calculated from the Stokes−Einstein relationship, $R = kT/6\pi\eta D$, where R is the Stokes radius of the molecule, D is the diffusion coefficient, k is the Boltzman constant, T is the temperature, and η is the solvent viscosity. Most measurements were taken using software version 12.3 (Particle Sizing Systems) in the intensity-weighted Gaussian distribution mode of the particle sizer. Light scattering measurements that were combined with the sedimentation coefficient data, calculated with analytical ultracentrifugation, were taken using volume-weighted Gaussian analysis. Typically, the laser beam was set at 500 mW, by diminishing the output beam with one or two neutral density filters. The photopulse rate was typically set at 400−600 kHz, and sufficient data were collected by 100 000 counts for channel 1 for intensity-averaged values and 400 000 counts for the volume-averaged values. The scattered light was measured at 90°, and the signal was sent to a 64-channel autocorrelator. For LDL, a 1.9 μs linear channel spacing was selected; when a single antibody was added to the LDL, the spacing was set at 3.5 μs for MB47 and 4G3, 3.8 μs for 1D1 and 2D8, and 3.8 μs for MB47 and 2D8.

LDLs, with and without monoclonal antibodies, were diluted to the desired concentration ([LDL] = 0.015 nM), and the antibody concentration was added in increasing amounts to reach the antibody equivalence point (total volume of 1.0 mL), with Mock buffer, which had been filtered through a 0.45 μm Millex HA filter unit (Millipore) to remove dust or other particulates. The diluted sample was allowed to equilibrate at 23 °C for 1 h and then transferred to a standard 1.0 cm quartz cuvette for dynamic light scattering, which was measured at 23 °C. At this temperature, Mock had a viscosity of 0.951 cP and a refractive index of 1.334. All measurements from a particular subject were performed in the same cuvette to avoid variations due to minor differences between cuvettes. However, cuvettes were always washed, rinsed thoroughly with filtered (0.45 μm) water, and dried with filtered air between samples.

Analytical Ultracentrifugation. Velocity sedimentation was performed in a Beckman Optima XL-A analytical ultracentrifuge (Beckman Instruments) at 40 000 rpm and 23 °C for 2 h. Scans were taken at 5 min intervals. The scans were analyzed at a wavelength of 230 nm. Sedimentation coefficients were calculated from measurements of the center of the symmetrical boundaries as a function of time and were reported as $s_{23,0.195MNaCl}$ in Svedbergs (10^{-13} s). All centrifugation runs were performed at the same concentrations as those in the dynamic light scattering experiments, and the solutions were diluted and mixed with Mock [0.195 M NaCl and 0.04% EDTA (pH 7.6) without azide] prior to the run and allowed to equilibrate at room temperature for 1 h. Analytical ultracentrifugation was performed on LDL, LDL bound to one monoclonal antibody, and LDL bound to two monoclonal antibodies.

Precision and Accuracy of the Measurements. The intensity-weighted (*z*-average) diffusion coefficient was given by a linear fit of the logarithm of the correlation function (minus the baseline) to the time, and this fit was established within the first 10−12 min of the experiment, at a count rate of 400 kHz (to a total of 100 000 counts in channel 1). The precision of the intensity-weighted diffusion coefficient for a standard LDL was 0.1 F, as determined from eight measurements over a period of 1 month, and individual measurements took 10−12 min and ranged between 1.95 and 1.97 F (lowest to highest value) with an average of 1.961 F for the standard LDL used in these experiments. The computer also estimated the distribution of Stokes radii from the small parabolic curvature of this line; determination of the curvature required about 4 times the number of counts. From the slope and curvature of the line, the standard deviation of a Gaussian curve was calculated. The distribution was shown as a Gaussian curve on a plot of the logarithm of particle size, and from this curve, the volume- and number-averaged particle sizes as well as the intensity-averaged particle size were calculated by the computer. The volume-averaged diffusion measurements recorded in Table 5 were averages of two independent, separate experiments, and the differences between these values were recorded as an estimation of the error. For these volume-averaged measurements, which required a longer time for equivalent accuracy, two 30 min readings were taken and, for the LDL, gave values of 2.14 and 2.12 F. When the average diffusion

Table 1: Monoclonal Antibodies Used To Generate LDL Complexes

antibody	epitope on apoB[a]	ref
1D1	474−539	*16*
2D8	1438−1480	*16*
4G3	2980−3084	*16*
MB47	3429−3453, 3507−3523	*13*

[a] Epitope on apoB gives the identity and length of the peptide which bound each antibody.

Table 2: Antibody Pairs Used To Generate Complexes

monoclonal pairs	approximate angle[a,b] (deg)
MB47 and 4G3	54
2D8 and 1D1	86
MB47 and 2D8	136

[a] The angles between the antibodies as measured on the LDL, as determined by electron microscopy (*4*−*6*). [b] These angles were estimated to within ±3°.

coefficient was combined with the sedimentation coefficient, a molecular mass of 2.68 kDa was obtained. In ref *21*, a classic paper on LDL hydrodynamics, the diffusion coefficient for a standard LDL, corrected with Mock buffer at 23 °C, of 2.06 F was obtained which, when combined with the sedimentation coefficient, yielded a molecular mass of 2.73 kDa. Thus, the similar diffusion coefficient and the closeness in molecular mass caused us to believe that our hydrodynamic measurements were reasonably accurate. The concentration dependence of the sedimentation coefficient was not expected to be noticeable at 20 μg/mL; it was not measured. The sedimentation measurements were analyzed by a least-squares fit through all 12 points, and 95% confidence limits were calculated from the deviation of the points from a straight line. In the last experiment described in Table 5 (for LDL, 2D8, and 1D1), for which the 95% confidence limits gave a precision of only 4.5%, this was accompanied by a definite, small bow to the log *r* versus *t* plot, with a slope which smoothly increased with time. In this particular run, a limited amount of dissociation was probably occurring behind the sedimenting boundary.

RESULTS

Addition of One Monoclonal Antibody to LDL. Four different monoclonal antibodies were selected, and the sequences recognized by these antibodies are given in Table 1. In Table 2 are the angles between the binding sites recognized by these antibodies, which were measured with electron microscopy. LDL at a constant concentration (0.015 nM) was titrated with each of the selected antibodies, to determine the maximum amount of LDL−Ab−LDL complex formed, which occurred at the peak of the titration curve and resulted in the largest increase in mean diameter. This was taken as the equivalence point. Once equivalence was reached, excess antibody caused antibody competition for the LDL dimers, resulting in a decrease in diameter due to the formation of single antibody−LDL complexes. A characteristic titration curve with a single monoclonal antibody and LDL is shown in Figure 2. In Table 3 are the intensity-averaged (*z*-averaged) diameters of the LDL dimers that resulted with each monoclonal at their respective equivalence points. Intensity-averaged diameters were calculated as 2

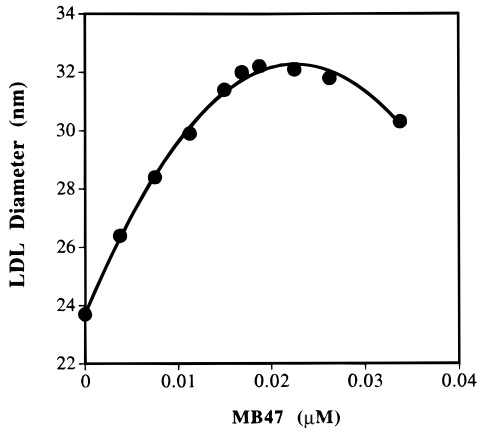

FIGURE 2: Titration of LDL with monoclonal antibody, MB47, to determine binding site equivalence. The concentration of LDL was kept constant at 0.015 μM. MB47 was added in increasing amounts from 0 to 0.04 μM. At each addition of antibody, the diameter was measured with light scattering. The diameter of LDL alone was 23.5 nm, and with increasing amounts of antibody, the apparent diameter increased until a maximum diameter of 32.0 nm was obtained for this antibody preparation. With very little antibody present, only a small amount of LDL dimer was formed, with the remaining LDL being unbound. As more antibody was added, more and more dimer formed, resulting in an increasing diameter which could be detected with light scattering, as shown. As increasing amounts of MB47 were added, there was a concentration at which almost all of the LDL was bound by antibody, the binding site equivalence point. The diameter began to decrease with excess antibody due to antibody competition, which results in one LDL bound only to MB47.

Table 3: Diameters of the LDL Dimers Made Using Each Antibody at Binding Site Equivalence

antibody	LDL (nm)[a] (monomer)	LDL and antibody (nm)[a] (dimer)	% increase in diameter
MB47	23.4 ± 3.9	33.0 ± 7.3	41.0
2D8	24.0 ± 3.9	35.0 ± 7.3	46.0
4G3	23.3 ± 4.0	34.9 ± 7.5	50.0
1D1	24.2 ± 4.0	35.1 ± 7.4	45.0

[a] Intensity-averaged diameter ± the standard deviation of the Gaussian particle size distribution.

times the Stokes radius, from the intensity-averaged diffusion coefficients. Although the percent increase in diameter for the dimer was different for each monoclonal antibody, this could be explained by differences in the dissociation constants of the antibodies, which suggested that even at equivalence of either 15 or 30 nM, only 85−95% of the antibody and LDL formed dimeric complexes.

Addition of Two Monoclonal Antibodies to LDL. After the equivalence points were determined for all the monoclonals, a second antibody was added, keeping both the LDL and the first monoclonal at a constant, equivalent concentration. Intensity-averaged diameters were measured for each of the three pairs of monoclonals. Figure 3A−C are the titration curves showing these diameters for 4G3 and MB47, 2D8 and 1D1, and 2D8 and MB47, respectively. Figure 3D shows the titration curves for all three pairs of antibodies, to compare the maximum diameters formed using two monoclonal antibodies. Each pair, characterized by a different angle between the monoclonal antibodies, showed a different maximum increase in diameter, ranging from 1.76 to 2.15 times that of the LDL alone. Once again, this increase could be attributed to the binding constants of each individual

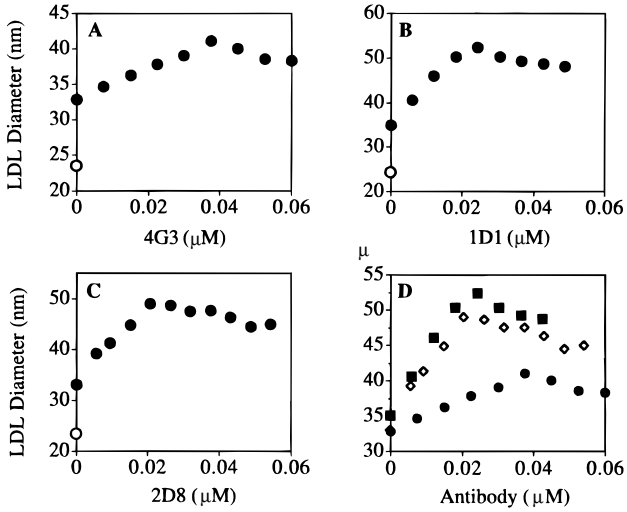

FIGURE 3: (A) Titration of LDL with monoclonal antibodies 4G3 and MB47. The white circle was for the diameter of LDL alone (23.5 nm), while the black circle immediately above was for the diameter of LDL and MB47 (32.8 nm) at 0 μM 4G3. The LDL and MB47 concentrations were kept at 0.015 μM. Increasing amounts of 4G3 (from 0 to 0.06 μM) were added. Binding site equivalence of LDL when both antibodies were added occurred at a maximum diameter of 41.0 nm. (B) Titration of LDL with monoclonal antibodies 2D8 and 1D1. The white circle was for the diameter of LDL alone (24.3 nm), while the black circle immediately above was for the maximum diameter of LDL and 2D8 (35.0 nm) at 0 μM 1D1. The LDL and 2D8 concentrations were kept at 0.015 uM. Increasing amounts of 1D1 (from 0 to 0.04874 μM) were added. Binding site equivalence of LDL when both antibodies were added occurred at a maximum diameter of 50.2 nm. (C) Titration of LDL with monoclonal antibodies 2D8 and MB47. The white circle was for the diameter of LDL alone (23.4 nm), while the black circle immediately above was for the diameter of LDL and MB47 (33.0 nm) at 0 μM 2D8. LDL and MB47 concentrations were kept at 0.015 uM. Increasing amounts of 2D8 (from 0 to 0.05437 μM) were added. Binding site equivalence of LDL when both antibodies were added occurred at a maximum diameter of 48.9 nm. (D) All three pairs of monoclonal antibodies form complexes with LDL. The black circles represent data for MB47 and 4G3. The black squares represent data for 2D8 and 1D1. The white diamonds represent data for MB47 and 2D8. Titration curves began at the respective diameter of the LDL dimer produced by the addition of only one monoclonal antibody. Both LDL and one monoclonal antibody were held at a constant concentration, while the second antibody was added in increasing amounts, to obtain a complex with a maximum diameter.

antibody, as well as to additional factors such as the angles between the antibodies, their orientation on the surface, and antibody flexibility and conformation. Again, after the second monoclonal antibody was added, a second equivalence point was obtained where the complex had its respective maximum diameter. At this point, all of the LDL was complexed to a maximum extent with the two different monoclonal antibodies, since it was known that LDL had only one apoB, and thus only one binding site for each different monoclonal

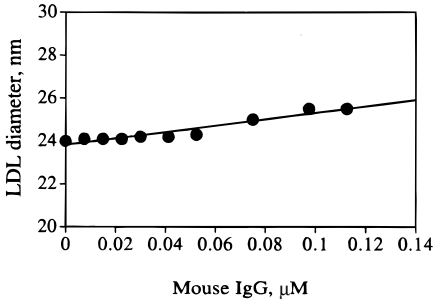

FIGURE 4: Effects of excess nonspecific antibody on LDL diameter. Purified mouse γ-globulin was added in a large, 4-fold excess of the usual equivalence point concentration of the monoclonal antibodies used. Mouse antibody was added from 0 to 0.1125 μM, and the diameter of LDL was measured with light scattering. The diameter only slightly changed from 24.1 to 25.5 nm at the greatest concentration of antibody.

antibody. In all cases in which a double titration was performed, a decrease in diameter was seen again after the second equivalence point, which corresponded to a decreased percentage of complexes due to dissociation by the excess antibody.

In Table 4 are the maximum intensity-averaged diameters measured with dynamic light scattering of all the complexes formed using one or two antibodies bound to LDL. Complexes as large as 52.3 nm in diameter were measured when two antibodies were added to LDL. The smallest complex formed with two monoclonal antibodies added had a diameter of 41.0 nm.

Effects of Adding Excess Antibody. The effect of adding a large excess of nonspecific antibody on the diameter of LDL was also investigated. Both rabbit and mouse IgG were used in this study at a 4-fold excess of the usual equivalence point concentration of the monoclonals. In the case of either nonspecific antibody, we saw little increase in the intensity-averaged diameter of the LDL, even at high concentrations, as shown in Figure 4 for the mouse antibody data. Thus, it is believed that the background concentration of antibody not bound to the LDL played an insignificant role in the diameter of the LDL we measured using dynamic light scattering.

Sedimentation Velocity Analytical Ultracentrifugation. To further characterize the complexes formed using different monoclonal antibody pairs, we used analytical ultracentrifugation. Sedimentation velocity experiments were performed with the LDL, with the LDL dimers formed by the single monoclonals, and with the LDL−antibody complexes formed by using two monoclonal antibodies at equivalence. To calculate sedimentation coefficients from the sedimentation profile, we measured the center of the symmetrical boundaries r and plotted ln r as a function of t. The slope of this line was equal to $\omega^2 s$. The sedimentation coefficients were reported as $s_{23,0.195\text{MNaCl}}$ in Svedbergs (10^{-13} s).

Table 4: Diameters[a] of LDL Complexes

	2D8 and 1D1 (nm)	2D8 and MB47 (nm)	MB47 and 4G3 (nm)
LDL alone	24.3[b]	23.4[b]	23.25[b]
LDL and one mAB	35.0 ± 7.0 (2D8)[c]	32.95 ± 7.2 (MB47)[c]	32.8 ± 8.6 (MB47)[c]
LDL and two mABs	52.3 ± 12.6	48.9 ± 10.9	41.0 ± 10.2

[a] Diameters measured by intensity-weighted Gaussian analysis, and followed by the standard deviation of the Gaussian curve. [b] Standard deviations in this row were inaccurate because of the short measurement time of 10 min; recorded values of the standard deviation of the Gaussian distributions were 6.0, 3.2, and 2.3 nm, respectively. [c] Dimer was formed using the antibody enclosed in parentheses.

Table 5: Molecular Mass and Frictional Ratios for the Average Size Complexes

mixture	$s_{23,NaCl}$ (S)[a]	D (cm^2/s) Ficks[b]	MW ($\times 10^6$ Da)	f/f_o	LDL[c]
LDL	5.18 ± 0.05	2.13 ± 0.014	2.68	1.06	1
LDL and MB47	10.02 ± 0.10	1.49 ± 0.035	5.70	1.18	2.08
LDL and 4G3	8.93 ± 0.05	1.52 ± 0.028	4.98	1.21	1.81
LDL and 2D8	9.40 ± 0.01	1.58 ± 0.013	5.04	1.16	1.83
LDL and 1D1	9.02 ± 0.08	1.60 ± 0.007	4.78	1.17	1.73
LDL, MB47, and 4G3	11.9 ± 0.17	1.43 ± 0.000	5.79	1.23	2.05
LDL, MB47, and 2D8	18.1 ± 0.01	1.10 ± 0.028	11.45	1.27	4.05
LDL, 2D8, and 1D1	17.1 ± 0.48	1.12 ± 0.014	10.62	1.28	3.75

[a] Ninety-five percent confidence interval calculated from the 12 points defining the straight line. [b] D_{ave} ± one-half of the difference between the two values of the diffusion coefficient recorded in two separate experiments. [c] Average number of LDLs in each complex.

Sedimentation velocity analytical ultracentrifugation gave complexes with *s* rates of 8.9, 9.0, 9.4, and 10.0 S, for the LDL dimers. For those complexes formed with two antibodies, *s* rates of 11.9, 17.1, and 18.1 S were found. By combining the sedimentation coefficient measured with analytical ultracentrifugation and the volume-averaged diffusion coefficient measured with dynamic light scattering, we were able to calculate the average molecular masses for each of these complexes and, thus, the number of LDLs in the complex. The volume-weighted average diffusion coefficients determined with light scattering are listed in Table 5. It was these values that were used in combination with sedimentation coefficients because sedimentation velocity analysis was also a volume-weighted average. In Table 5 are the measured diffusion and sedimentation coefficients, the calculated molecular masses of each complex, and the number of LDLs per complex, along with the frictional coefficients calculated for each complex formed. The frictional coefficients ranged from 1.06 for the dimers to 1.28 for the larger complexes formed. All the LDL−single antibody complexes appeared to form dimers with about two LDLs per complex. Those dimers with less than two LDLs per complex indicated that some of the complexes were partially dissociated. When two different monoclonal antibodies were used to form the larger complexes, the size of the complexes varied from about two to four LDLs per complex.

Heterogeneity. Two plots showing heterogeneity are shown in Figure 5 for LDL by itself and for LDL, MB47, and 2D8, our largest complex. These plots were quite reproducible when taken after 30 min of run time, and showed the degree of heterogeneity in size in a Gaussian bar graph versus the logarithm of the antibody diameter. Heterogeneity plots for the other 30 min time points, representing various mixtures of LDL and monoclonals, were similar to these curves shown in Figure 5. Some estimate of reproducibility also was given by the χ^2 value, which was less than 1 in both cases shown here, and in most cases in Figure 5, representing a very good fit of the experimental data to a Gaussian curve.

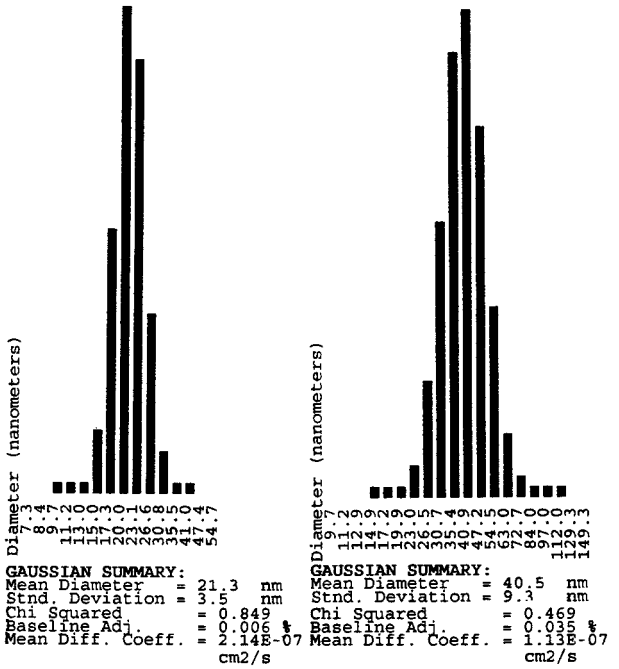

FIGURE 5: Volume-weighted Gaussian analyses are provided for LDL (left-hand bar graph) and LDL, MB47, and 2D8 (right-hand bar graph). Data were collected for 30 min and 24 s (LDL) and for 32 min and 38 s (LDL, BM47, and 2D8) at a count rate of about 600 kHz, by which time the standard deviations of the Gaussian curves remained significantly unchanged. The deviations shown were typical of all of the data presented in Table 5.

DISCUSSION

Qualitative Analysis. Many of the questions previously formulated in the introductory section have been answered, at least in a qualitative sense, by the results already presented. Thus, it has been shown that essentially all of the LDLs were bound by these monoclonal antibodies. Even the mixtures composed of two antibodies and LDL showed almost complete binding in the analytical ultracentrifuge.

Precipitation did not occur with one antibody because a single antibody could form only a dimer. With two antibodies, a finite chain or an intact, circular ring was more probable. Indeed, circular complexes were favored entropically, because ring closure did not require the addition of another antibody or another LDL, unlike lengthening a daisy chain. Moreover, as assembly continued, the concentration of free antibody and LDL dropped drastically, until the lower concentration was unable to maintain a chain free of breaks. Indeed, we found that at concentrations much higher than those employed in the electron micrographs very finite sized groups of tetramers or dimers predominated.

Determination of Diffusion Coefficients. In using dynamic light scattering, the particle diameter was determined from the diffusion coefficient. Thus, for spheres, the diffusion coefficient was inversely proportional to the radius, as described by the Stokes−Einstein relation:

$$D = kT/f, \text{ where } f = 6\pi\eta R_h(f/f_o) \quad (1)$$

where R_h is the radius of the unsolvated particle molded into

a sphere, f is the frictional coefficient of the actual molecule, f_o is the frictional coefficient of the unhydrated sphere, and f/f_o is the frictional ratio, measured as 1.11 (*21*). Thus, the diffusion coefficient for a sphere would decrease as the sphere increased in size.

With the Addition of a Single Monoclonal Antibody to LDL, How Would the Diffusion Coefficient Change? Since monoclonal antibodies are bivalent, they have two binding sites for apoB. Since there is only one apoB per LDL molecule, the antibody would be expected to bind two separate apoBs on two different LDLs. Therefore, the antibody would cause the formation of LDL dimers held together by a single antibody molecule. This complex would have a smaller diffusion coefficient than a single LDL, and this could be measured with dynamic light scattering. By how much would the diffusion coefficient be expected to decrease? An approximate method for computing the frictional ratio, f/f_m, of a structure composed of N identical spherical subunits with a radius R was developed by J. G. Kirkwood and J. Riseman, as described in ref *22*. Their eq 10-34 is

$$f/f_m = N(1 + 1/N \sum_i \sum_{j \neq 1} \alpha_{ij}^{-1})^{-1} \quad (2)$$

where f is the frictional coefficient of the complex, f_m is the frictional coefficient of the monomer, and $\alpha_{ij} = a_{ij}/R$, where a_{ij} is the distance between the centers of spheres i and j.

Calculation of Expected Values for Monomeric and Dimeric Lipoproteins. LDLs contain about 20% apoB by weight, and apoB is virtually their only protein. Because the molecular mass of apoB is known, the molecular mass of LDL is readily approximated as 513 000 g mol^{-1}/0.20 = 2.6 $\times$ 10^6 g mol^{-1}. With a density of 1.032 g/mL, the radius, assuming an unhydrated sphere, is 10 nm.

Experimentally determined radii, often called Stokes radii, differ from unhydrated sphere radii by the frictional ratio. The frictional ratio has been measured as 1.11 for LDL (*21*). When this correction was applied to the estimated unhydrated radius of the LDL sphere, the calculated Stokes radius became 11.1 nm, and the Stokes diameter became 22.2 nm. Because LDLs were heterogeneous in size, however, this value represented an averaged diameter, and in this case, the number-average diameter. Dynamic light scattering using the intensity-weighted analysis mode of the particular algorithm employed with the software supplied by Particle Sizing Inc. yielded an intensity-average diameter. The intensity-average diameter was approximately given by eq 3 below. Equations 4 and 5 approximate the volume-average diameter and the number-average diameter, respectively:

$$D_Z^3 = \frac{\sum C_i D_i^6}{\sum C_i D_i^3} \quad (3)$$

$$D_W^3 = \frac{\sum C_i D_i^3}{\sum C_i} \quad (4)$$

$$D_N^3 = \frac{\sum N_i D_i^3}{\sum N_i} \quad (5)$$

where D is the particle diameter, C is the mass concentration, and N is the number of particles of size i. For a heterogeneous distribution of particle sizes, the intensity-averaged diameter was always larger than the number- or volume-averaged diameter, and values of 23.0−24.0 nm generally found for LDL diameters with this technique seemed reasonable.

From eq 10-34 of ref *22*, when $N = 2$ and $\sum_i \sum_{j \neq i} a_{ij}^{-1} = 2R/a$, the previous equation reduces to a simple expression. These two spheres would have a Stokes radius given by

$$f/f_m = \frac{2a}{a + R} \quad (6)$$

where f is the frictional coefficient of two spheres, f_m is the frictional coefficient of one sphere, a is the distance between the centers of the two spheres, and R is the radius of one sphere. When a is equal to $2R$, then the two spheres touch, and f is equal to $1.333 f_m$. However, the addition of an antibody may separate the spheres by an additional distance. If the angle between the antibody arms opens to 180°, then a could be as large as $4R$, and using the previous equation, $f = 1.6 f_m$. Thus, the increase in f could be between 33 and 60%. The distance between the spheres will vary depending on many factors such as the inclination of the antibody arms at the binding site, the degree of attraction or repulsion between the LDL spheres, antibody flexibility, and antibody binding affinities. Antibody binding affinities will influence the measured diameter greatly. If a small percentage of LDL exists as a monomer instead of as a dimer, then the increase in average diameter and the frictional coefficient would be smaller than expected. And this was probably occurring with the antibodies used in this study.

Upon addition of monoclonal antibody MB47 to our LDL preparations, we saw maximum percent increases in diameter of about 33 ± 1.0%, which was close to the theoretically estimated value for touching spheres, suggesting that at equivalence, 15 nM LDL, essentially all of the LDL formed a dimer with this antibody. But this was probably not the correct interpretation, because upon addition of antibody, the two LDLs were not expected to form 100% dimer. If we assumed that MB47 formed only 90% dimer, then the actual increase in the frictional ratio should be about 37% (or an f/f_m of 1.37) for the dimer and 0% (or an f/f_m of 1.00) for the 10% monomer, to average 33.3% (or an f/f_m of 1.333), which was seen in our experiments. Moreover, the distance separating the spheres would increase by about 10%, thus going from an a of $2R$ to an a of $2.2R$, causing the frictional coefficient to increase from 33.3 to 37%, according to the eq 6.

Determination of Molecular Masses. To characterize our system more accurately, another hydrodynamic technique, analytical ultracentrifugation, was employed to determine the molecular masses of the complexes formed by the addition of two monoclonal antibodies. The molecular masses (M) were calculated with the equation

$$(s/D) = [M(1 - \upsilon\rho)]/(RT)$$

where s is the sedimentation coefficient, D is the diffusion coefficient, $\bar{v}$ is the partial specific volume, $\rho = \rho_{mock} = 1.009$ g/mL, R is the gas constant (8.314 $\times$ 10^7 erg mol^{-1} °C^{-1}), and T is 296.15 K. From the molecular mass, it was possible to determine the number of LDLs in each complex. And indeed, the other monoclonal antibodies used in these

studies (2D8, 4G3, and 1D1) were shown to form complexes having a little less than two LDLs per complex. The data generated from these two studies showed us that we have characterized our system properly.

Binding of LDL to One Monoclonal Antibody. The measured molecular masses for the antibody dimers are shown in Table 5. For all antibodies, with the exception of MB47, these values were all a little less than that of the theoretical complex formed between one antibody and two LDLs. This result was believed to be caused by a little dissociation occurring at equivalence. Beyond equivalence, the molecular masses began to drop due to antibody-driven dissociation. If we ignore any experimental error at equivalence and attribute all the loss in molecular mass to dissociation, the decrease varied from one antibody to the next, but it was on the order of 10−20%. This seemed reasonable, for if the dissociation constant were 2 orders of magnitude lower than the concentration, that is, 0.3 nM, then 90.5% of the binding sites would be filled, making the probability that both sites would be filled equal to 0.905^2, or 81.9%. Thus, 1 − 0.819 or 18.1% of the antibody would have one empty site. If the dissociation constant were 3 orders of magnitude lower than the concentration, that is, 0.03 nM, then the probability that a single antibody site would be filled would be 96.9% and that both sites were filled would be 0.969^2, or about 93.8%. Thus 1 − 0.938 or 6.2% of the antibody would have one empty site. In fact, a quadratic formula gives the probability that a single antibody binding site is filled:

$$p = \frac{(x_o + y_o + K) - \sqrt{(x_o + y_o + K)^2 - 4x_o y_o}}{2y_o} \quad (7)$$

where p is the probability of filling an antibody site when x_o is the total molarity of LDL added, y_o is the total molarity of antibody sites added, and K is the molarity of the dissociation constant. The values of x_o and y_o are known at equivalence. Therefore, it is possible to calculate K, the dissociation constant for these complexes, from the value of p, using eq 7. The values of K ranged from 10^{-12} to 10^{-10} M, and these values appeared to be quite reasonable for monoclonal antibodies.

Of course, it cannot be true that the binding constant is quite the same for the first LDL bound and the second LDL bound. In fact, in many conformations of antibody with one LDL bound, filling the second site would result in a second LDL which occupied the same space as the first LDL; such overlapping conformations are forbidden. Thus, the probability that the second site could be filled is reduced probably by a factor of ≥2. However, it must be exactly true that the calculated average p is equal to $(p_1 p_2)^{1/2}$, where p_1 and p_2 are the values for the binding of the first and second LDL to the antibody, respectively. Another way of looking at this problem is that the actual dissociation constant will be a little larger (less tight) for the first of the two LDLs released.

Binding of LDL to Two Monoclonal Antibodies. Molecular masses for the larger complexes formed when two monoclonal antibodies were added to the LDL are shown in Table 5. The data showed a dimer formed by two monoclonal antibodies separated by 54° on the surface of the two LDLs. In this case, we believe that a cyclic dimer was easily formed. Cyclic dimer formation did two things. It effectively increased the binding constant of the antibodies in the complex, for the first "dissociation" reaction simply opened the ring, and the probability of the ring reclosing was usually much greater than the probability of a second dissociation. Second, cyclic dimer formation stopped further growth; that is, it terminated increased expansion since all of the antibody binding sites and all of the LDL binding sites were occupied. This suggested that a cyclic complex had been formed when the antibodies were separated by 54°. And from the molecular mass, we see that this predominant species was a dimer.

The titration curves in panels B and C of Figure 3, those curves with 2D8 and 1D1, and 2D8 with MB47, respectively, showed those monoclonals formed complexes with about twice the molecular mass of that from the curve from LDL with MB47 and 4G3. These complexes must have been mostly tetramers with only a small fraction of dimers, trimers, or pentamers. These larger complexes were seen in the electron micrographs, although since the complexes were diluted by another order of magnitude before spreading, the average size of complexes would have decreased.

These data which determined the molecular mass and number of LDLs in each complex verified the light scattering data. In the light scattering experiments, we only believed that we were forming dimers and tetramers on the basis of hydrodynamic theory, but now with the analytical ultracentrifuge, we have shown that indeed almost all complexes that were formed with one monoclonal antibody were LDL dimers, with only a small amount of dissociation at the equivalence point. Moreover, our data indicated that indeed different pairs of monoclonal antibodies which were separated by different angles formed complexes of different sizes. Thus, the number of LDLs in a complex will vary due to many factors, and especially due to the binding affinity which could result in a smaller diameter than that originally estimated. This study of complexes formed between bivalent antibodies and LDL provides us with some assurance that our dynamic light scattering system is relatively well understood.

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