A single copy of apolipoprotein B-48 is present on the human chylomicron remnant

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Abstract Individuals homozygous for the e2 allele encoding apolipoprotein E exhibit a remnant removal defect and accumulate substantial levels of intestinally derived particles containing apolipoprotein B-48 (apoB-48). Such lipoproteins were isolated from the plasma of E2/E2 individuals, and further purified by affinity chromatography using a polyclonal antibody specific for selective binding and removal of apoB-100-containing lipoproteins. The unbound lipoproteins, termed chylomicron remnants, were particles with average hydrated diameters of 31.2 nm as determined by dynamic light scattering. They contained apoB-48 and ApoE as their only protein components. The number of apoB-48 molecules on each lipoprotein was assessed by counting the number of antibody molecules bound to the surface of the chylomicron remnants, using either a monoclonal antibody specific for a single epitope on apoB-48 or a mixture of two such monoclonal antibodies specific for widely separated epitopes. The results of this analysis seem unambiguous: no more than one apoB-48 resides on the chylomicron remnant. Because apoB appears to be unable to transfer among lipoprotein particles, it may be inferred that nascent chylomicrons also contain a single copy of apoB-48.—**Phillips, M. L., C. Pullinger, I. Kroes, J. Kroes, D. A. Hardman, G. Chen, L. K. Curtiss, M. M. Gutierrez, J. P. Kane, and V. N. Schumaker.** A single copy of apolipoprotein B-48 is present on the human chylomicron remnant. *J. Lipid Res.* 1997. **38:** 1170–1177.

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The question of the number of apoB-48 molecules residing upon the human chylomicron has remained unanswered, although it now seems established that a single apoB-48 or apoB-100 is present on rat VLDL. The question is important, for if the human chylomicron contains more than a single copy of apoB-48, the mechanism of chylomicron biosynthesis would be different from that of the rat apoB-48-containing VLDL, which requires only a single molecule of apoB-48 for its formation.

In an early study (1) the average apoB content of VLDL was measured and found to be the same as the average apoB content of LDL. Determination of the number of separate apoB molecules on the LDL was a more difficult problem, however. The large, hydrophobic apoB polypeptide required 6 M guanidinium hydrochloride for solubilization in an unaggregated state. In the presence of this chaotrope, non-ideality resulted in an underestimation of the molecular weight by a factor of 1/2, so that Smith, Dawson, and Tanford (2) concluded that there were two apoB molecules on LDL. When apoB-100 was cloned and sequenced in 1986, it was apparent from its high molecular weight that there could be but a single apoB-100 molecule on LDL (for reviews see 3, 4). This conclusion was subsequently extended to the VLDL by accurately determining particle sizes on tightly defined fractions of VLDL together with measuring apoB molecules by a radioimmunoassay, which determined the number of apoB molecules irrespective of whether they were apoB-48 or apoB-100 (5). Moreover, because the rat liver, unlike the human liver, secreted both apoB-100 and apoB-48-containing VLDL, it could be concluded that a single apoB-48 molecule was sufficient for the biosynthesis of apoB-48-containing rat VLDL. More recent molecular studies using permanent hepatocyte cell lines also support the single apoB molecule hypothesis for VLDL by showing that a single apoB molecule is required for the co-translational formation of a primary lipoprotein particle in the lumen

Abbreviations: apoB-48, apolipoprotein B-48; apoB-100, apolipoprotein B-100; EDTA, ethylenediaminetetraacetic acid; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

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of the endoplasmic reticulum (6, 7), and that for apoB-48-containing particles, additional lipid is subsequently acquired in a second step to form the nascent VLDL (8). From these results, it also may be inferred that a single apoB-48 molecule might suffice for chylomicron formation as well.

The inference that a single apoB-48 molecule is sufficient for chylomicron formation, however, runs counter to the data of Bhattachayra and Redgrave (9). These authors measured the apoB content of human chylomicrons after specific precipitation of apoB molecules with tetramethyl urea, and determined chylomicron particle number by electron microscopy. Their value of 470 kDa of apoB-48 per chylomicron was close to the amount expected if two molecules of apoB-48 were present on human chylomicrons.

In this communication we report a different approach to the measurement of the apoB-48 content of the chylomicron. Whereas the concentration of chylomicron remnants in normal plasma is very low due to rapid endocytosis in the liver, we took advantage of the accumulation of chylomicron remnant particles in individuals with familial dysbetalipoproteinemia (10). These individuals are homozygous for apolipoprotein E2, a form of apoE that is ligand-defective for LDL receptor binding. Chylomicron remnants also afforded an advantage for an immunoelectron microscopic approach because they are much more homogeneous in diameter, smaller, and deform less than nascent chylomicrons. The apoB-48-containing remnant particles can be purified by removing apoB-100-containing particles by affinity chromatography using antibodies directed to the carboxy terminal region of apoB-100 that is absent from the truncated gene product, apoB-48. In contrast to the macroscopic approach used by Bhattacharya and Redgrave (9), we have counted the numbers of anti-apoB-48-specific monoclonal antibodies bound to individual remnant particles. Our data strongly indicate the presence of no more than a single apoB-48 molecule on the human chylomicron remnant.

MATERIALS AND METHODS

Isolation and purification of chylomicron remnants

Three volunteer donors contributed blood for this study. Each had apparent homozygosity for apolipoprotein E2 based on isoelectric focusing of the apolipoproteins of the $d < 1.006$ g/ml supernatant fraction (11). Sixty milliliters of blood was drawn into tubes containing preservatives, such that the final concentrations were: EDTA, 0.04%; sodium azide, 0.05%; benzamidine, 0.03%; gentamycin, 80 mg/l. These agents were also maintained in buffers used in subsequent steps of purification. Plasma was separated at 4°C and the solvent density was raised to 1.019 g/cm^3 by the addition of D_2O. The plasma was centrifuged for 24 h at 105,000 *g* in a Beckman 40.3 rotor. The supernatant portion was aspirated and subjected to a second ultracentrifugation for 24 h at $d = 1.019$ g/ml (12). The supernatant portion, which contained both apoB-100-containing lipoproteins and apoB-48-containing chylomicron remnants, was passed over affinity columns containing polyclonal antibodies to carboxy terminal sequences of apoB-100.

Preparation of anti-human apoB-100 antibodies

Human apoB-100 antiserum was prepared using two inducible TrpE fusion protein antigens grown in *E. coli*. Human genomic DNA was amplified by PCR with the oligonucleotides: 5′CTCACCATATTCAAAACTGA GTTGAGGG3′ (sense; nucleotides 12234–12261) and 5′ATTTGTTCCTCCTCCCCCAAGTTTAGC3′ (antisense; nucleotides 14069–14095). For the "4081" fusion protein (apoB codons 4081 to 4342, apoB-90.0 to apoB-95.7), a Pst/HindIII fragment (nucleotides 12450–13234) was ligated into pATH20 (ATCC no. 37700); for the "4342" fusion protein (apoB codons 4342 to 4525, apoB-95.7 to apoB-99.8), a HindIII fragment (nucleotides 13234–13785) was ligated into pATH21 (ATCC no. 37701). Each was transformed into *E. coli* strain RRI. Positive clones were induced and expressed proteins were examined by SDS-PAGE. Electrotransfer blots were probed with a rabbit antiserum to human LDL.

Recombinant plasmids were expressed as previously described (13). TrpE-apoB fusion proteins were purified by electrophoresis in preparative 9% SDS polyacrylamide gels. Goats were injected with 100 μg of protein at monthly intervals and bled after 4 months. Specificity of the antiserum was tested using immunoblots of SDS-PAGE gels of delipidated human LDL. When a sufficiently high titer was achieved, the animals were plasmapheresed.

The antibodies were purified by affinity chromatography on a low density lipoprotein affinity column constructed by binding centrifugally isolated LDL ($1.055 > d > 1.020$ g/ml) to CNBr-activated Sepharose 4B (Pharmacia). The antibodies were eluted from the LDL column with 0.3 M acetic acid, 75 mM NaCl, pH 3, and were used to construct the selective C-terminal affinity columns by covalent linking to CNBr-activated Sepharose 4B. The C-terminal columns were operated with a binding buffer composed of 0.5 M NaCl, 5 mM Tris, 1 mM EDTA, 0.05% NaN_3, pH 7.4. The '4081' column was used to remove the bulk of the apoB-100-containing

particles. Subsequently the '4342' column was used to remove the small amount of residual apoB-100 particles that were associated with the chylomicron remnant preparation.

Quantitative removal of apoB-100 was judged by SDS gel electrophoresis. Proteins were visualized by silver staining using the Bio-Rad Silver Stain Kit (Bio-Rad, Richmond, CA). Only apoB-48 and apoE proteins could be detected after passage through the two columns. The remnant lipoproteins containing apoB-48 and apoE proteins were concentrated against 0.15 M NaCl, 5 mM Tris, pH 7.4, by negative pressure protein dialysis/ultrafiltration using a Micro-ProDiCon Filtrations System (Spectrum, Houston, TX).

Electron microscopy

To determine the number of apoB-48 molecules, chylomicron remnants were incubated with one or two monoclonal antibodies at concentrations of about 25 nM apoB-48, where apoB-48 was estimated as two-thirds of the total lipoprotein protein, and 100 nM for each antibody. Incubations were in 0.15 M NaCl, 10 mM Tris, 1 mM EDTA, pH 7.4, for 60–120 min at 0°C. Samples were then diluted 50-fold with 0.196 M NaCl, 1 mM EDTA, pH 7, and spread immediately. Grid preparation was by the single carbon technique (14), as described previously (15), with the following modifications: the sample was allowed to adhere to the carbon for 15–20 sec; the water wash was deleted; and the sample was stained for 30–45 sec with 1% uranyl acetate. A separate dilution was used for each grid, and less than 75 sec was required for the multistep process of diluting the concentrated solution, absorbing the particles to the carbon film, transferring the film to the stain, picking up the film with the grid, and removing the excess liquid. The dissociation constants for these antibodies probably lie in the range between 10^{-8} to 10^{-9} M; for example, the dissociation constant for 2D8 binding to VLDL has been measured as 1 nM, (16), and although some dissociation undoubtedly occurred during the 75 sec required for grid preparation, 30 to 40% of the remnant particles were observed to bind antibodies, a percentage suitably high to proceed with these studies. After air drying, grids were examined at an apparent magnification of 30,000 in a Hitachi H-7000 electron microscope operating at 75 kV using a 200 μm condenser aperture and a 50 μm objective aperture. The microscope magnification was calibrated using a diffraction grating replica (54,800 lines/inch).

Photographs were taken of areas on the electron microscope grid where the stain was sufficiently thick so as to allow simultaneous visualization of spherical (circular) chylomicron remnants and antibodies in the background. For measuring the number of antibodies bound, the negatives were magnified 2.8-fold onto Kodak Ektamatic SC paper at a contrast grade of 4, and the enlarged prints were scored. The central angle between pairs of monoclonal antibodies binding simultaneously to a single chylomicron remnant was measured when the chylomicron remnant was circular in projection. Angles were measured as previously described (15, 17).

For measurement of the nonspecific background, mixtures of chylomicron remnants, 25 nM in apoB-48, with a monoclonal antibody without specificity for any lipoprotein component, were made, with the nonspecific monoclonal antibody either 100 nM, to emulate the binding mixtures involving one monoclonal antibody, or 200 nM, to emulate the binding mixtures using two monoclonal antibodies. These mixtures were incubated, mounted for electron microscopy, and analyzed in the same fashion as the specific monoclonal antibody mixtures.

Dynamic laser light scattering

Light scattering measurements were done using a Nicomp 370 Submicron Particle Sizer to measure the autocorrelation function from fluctuations in scattering intensity, and a Stabilite 2017, 6 watt Argon Laser from Spectra Physics Lasers, San Francisco, CA, as an external light source. The 514.5 nanometer wavelength laser line was used, and the laser was run at a current of 900 mw, diminished prior to entering the sample by two neutral density filters. Data collection was done to either 381,000 or 201,000 counts in duplicate runs using a photopulse rate of 360 KHz and a 5.3 microsecond channel width. The autocorrelator software was used to produce a volume-weighted diameter to avoid overrepresentation of larger, more intensely scattering particles.

RESULTS

Isolation and characterization of chylomicron remnant particles

After passage through the two different C-terminal immunoaffinity columns, no apoB-100 band was detectable on SDS gel electrophoresis and the apoB-48 band was the sole high molecular weight component observed (**Fig. 1**). The particle size distribution obtained on the B-48-containing particles by dynamic light scattering yielded a volume-averaged, hydrated diameter of 31.2 nm, obtained with software provided by the manufacturer (Particle Sizing Systems, Palo Alto, CA). This value is the Stokes diameter derived from the diffusion constant for these particles, the quantity actually measured by dynamic light scattering.

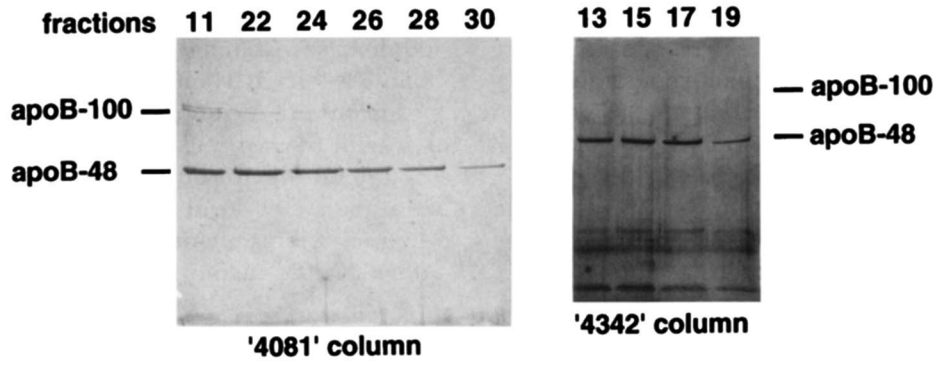

Fig. 1. Silver stained SDS-PAGE gels of apoB-48 and apoB-100. A small amount of apoB-100 is still present in fractions eluted from the '4081' column. When this material is further purified on the '4342' column, however, the apoB-100 protein is no longer seen.

Visualization of monoclonal antibodies bound to chylomicron remnant particles

The five monoclonal antibodies used in these studies are listed in **Table 1** along with the approximate locations of their specific epitopes on the primary sequence of apoB.

A representative electron micrograph is shown in **Fig. 2** illustrating the binding of a pair of monoclonal antibodies to individual lipoproteins (arrows) when B4 and 2D8 were simultaneously added to the chylomicron remnants. For negative contrast, a thin layer of uranyl acetate stain was used so that the antibody molecules were visualized as well as the lipoproteins; when the thicker potassium phosphotungstate stain was used, the lipoproteins were visible but the antibodies often could not be seen. As we have previously reported for LDL (15), the lipoprotein particles were flattened when the thinner uranyl acetate stain was used and their apparent diameters now averaged about 350 nm. In a typical experiment the value was 346Å ± 22Å (standard deviation).

Determination of the number of copies of apoB-48 on the chylomicron remnant particles

The results from two separate experiments utilizing two different preparations of chylomicron remnants are shown in **Table 2** and **Table 3.**

TABLE 1. Locations of the epitopes recognized by the monoclonal antibodies on the primary sequence of apoB

Monoclonal Antibody	Epitope Location	Epitope Location as Percent of Length of ApoB-48
MB19	71	3
MB24	405–539	22
MB11	1022–1031	48
2D8	1438–1480	68
B4	1854–1878	87

Production and characterization of the antibodies is described in references 16, 19, 20, 21, and 22. Epitope locations are taken from references 21 and 22.

In the first study, 709 remnants were examined. In the second column of Table 2 for monoclonal antibody 2D8, it may be seen that 172 out of 254 remnant particles were apparently devoid of antibody, while 80 lipoprotein particles, or 31%, were observed to bind a single antibody molecule, and two remnants, or less than 1%, were each associated with two antibody molecules. However, the number of antibody molecules actually bound to remnants is probably much greater than the number observed to bind, because when superimposed on top or beneath the remnants, the antibodies lack sufficient contrast to be resolved. For example, it may be calculated from the known dissociation constant of 2D8 for VLDL (18) that 95% of the specific epitope should bind antibody prior to dilution. Assuming a reasonable half-life of 10 min, it may be calculated that less than 10% of this bound antibody would be lost during the 1.25 min required to prepare the grid.

Column 3 of Table 2 shows a similar pattern of binding with the second monoclonal B4, except that an even higher percentage of the remnants was observed to bind a single antibody molecule, 38%, while only one remnant particle was seen associated with two antibody molecules.

Column four of Table 2 shows the results of an experiment in which the two monoclonals were simultaneously incubated with chylomicron remnants at the same concentrations of each antibody as before. Now the percentage of remnant particles seen to bind two monoclonal antibodies has jumped dramatically from 1% to 36%. Only 1% (two remnants) of the particles was observed with three associated monoclonal antibodies.

The experiment shown in Table 2 was repeated using a second preparation of chylomicron remnants. Four different single monoclonal antibodies and three pairs of the monoclonal antibodies were incubated with this preparation. These data are shown in Table 3. As may be seen from an inspection of the latter data, very simi-

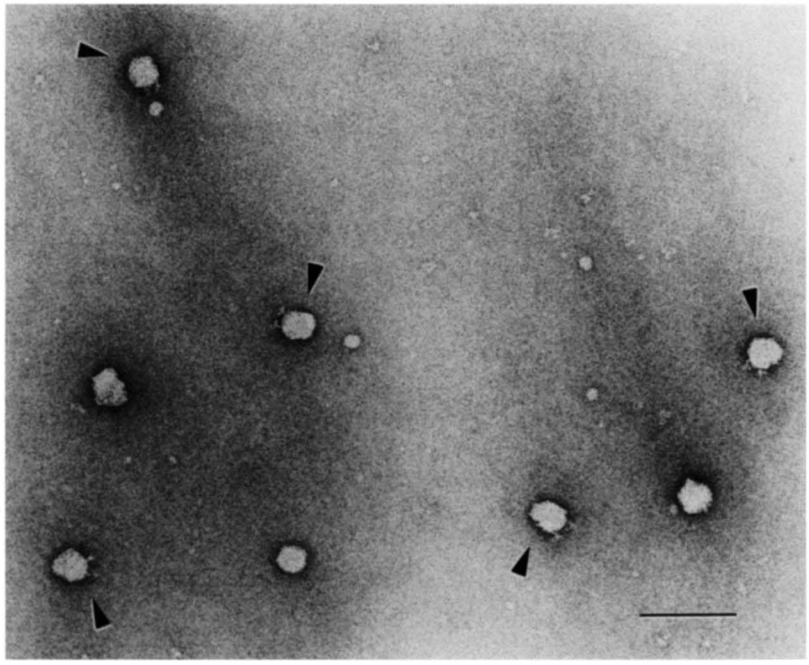

Fig. 2. Electron micrograph of pairs of monoclonal antibodies binding to chylomicron remnants. Monoclonal antibodies 2D8 and B4 were added to chylomicron remnants, as described in the text, and observed using a thin layer of uranyl acetate negative contrast stain. The arrowheads point to remnant particles appearing to bind two monoclonal antibodies.

lar results were obtained. When single monoclonal antibodies were used, 56–64% of the remnant particles appeared to bind no antibodies, while 35–40% were seen to bind a single antibody and 1–5% apparently bound two antibody molecules. When antibodies were added in pairs, 36–44% of the remnant particles bound two monoclonal antibodies, while 1 or 2% apparently bound three. None bound four.

What is the probability that some of the apparent binding actually represents accidental superposition of an Fab or Fc with the periphery of a remnant particle? By spreading an anti-C1q (non-specific for apolipoproteins) monoclonal antibody with these chylomicron remnants at 1- and 2-fold the indicated concentration, we have determined that approximately 4% of the antibodies actually used would be close enough to the remnants to be considered bound, without specific binding (data not shown). This would satisfactorily explain the few excess particles in Table 2 and 3.

TABLE 2. Number of chylomicron remnants observed to bind 0, 1, 2, and 3 monoclonal antibodies 2D8 and B4

Number of Bound Mab	2D8 (only)	B4 (only)	2D8 + B4 (added together)
0	68 (172)	61 (67)	52 (181)
1	31 (80)	38 (42)	11 (38)
2	1 (2)	1 (1)	36 (124)
3			1 (2)

Monoclonal antibodies were added to remnants as the only antibody present, or both antibodies were added together, at concentrations of 100 nM of each antibody and 25 nM of chylomicron remnants. After incubation at 0°C, the solution was diluted 50-fold and immediately applied to the grid and stained. Forty-two fields from three grids were scored. Eighteen molecules that were either too distorted or too buried in stain to be scored were discarded, leaving 709 remnants counted. Percent of remnants is listed, and the number of remnants observed with 0, 1, 2, or 3 monoclonals bound is in parentheses.

Distribution of angles

There is a second approach to the question of the number of apoB-48 molecules residing upon each chylomicron remnant particle that is portrayed in **Fig. 3.** Panel A shows a chylomicron bearing a single apoB-48; when two monoclonals bind to this particle, there is only one small angle formed between them, and a plot of the angular distribution will show a single, sharp peak. On the other hand, if there are two apoB-48 molecules on the chylomicron, then there are four possible angles which could be measured when two monoclonal antibodies bind, as shown in panel B, and a plot of the angular distribution will show multiple peaks, depending upon the relative abundances of the various angular species.

The results of the actual measurements of the angles between pairs of the different monoclonal antibodies are shown in **Fig. 4.** In each case, single, well-defined angular distributions were formed, clearly indicating that a single apoB-48 was being studied.

Chylomicron remnant particles bind all four monoclonal antibodies simultaneously

To show that all of the monoclonal antibodies could simultaneously bind to the chylomicron remnant, we replaced monoclonal MB24, which binds at residue

TABLE 3. Number of chylomicron remnants observed to bind 0, 1, 2, and 3 monoclonal antibodies 2D8, B4, MB11 and MB24

Number of Bound Mab	2D8	B4	MB11	MB24	2D8 + B4	2D8 + MB11	B4 + MB11
0	61 (104)	56 (127)	64 (133)	58 (91)	51 (131)	48 (57)	50 (97)
1	38 (65)	40 (90)	35 (72)	37 (59)	12 (31)	8 (9)	12 (23)
2	1 (1)	4 (10)	1 (3)	5 (8)	36 (92)	44 (53)	36 (70)
3					1 (2)	1 (1)	2 (4)

Monoclonal antibodies were added to remnants as the only antibody present, or both antibodies were added together, at concentrations of 100 nM of each antibody and 25 nM of chylomicron remnants. After incubation at 0°C, the solution was diluted 50-fold and immediately applied to the grid and stained. Percent of remnants is listed and the number of remnants observed with 0, 1, 2, or 3 monoclonals bound is in parentheses.

405–539, with MB19, which binds at residue 71. This substitution places all of the epitopes on the same arc, if apoB-48 has a similar structure to that of apoB-100 on LDL (17). The experiment was then attempted with 65 chylomicron remnants, and to our surprise almost one molecule out of three (20 out of 65) of the remnant particles bound all of the monoclonal antibodies. This is shown in **Fig. 5,** where 4 molecules were selected from the group of 20 to show this effect. Of the remaining 45 molecules in this group of 65, 21 bound no monoclonals, 9 bound a single monoclonal, 4 bound to two monoclonals, 10 bound to three monoclonals, and 1 bound five monoclonals. These data are also consistent with there being a single apoB-48 on the chylomicron remnant particle.

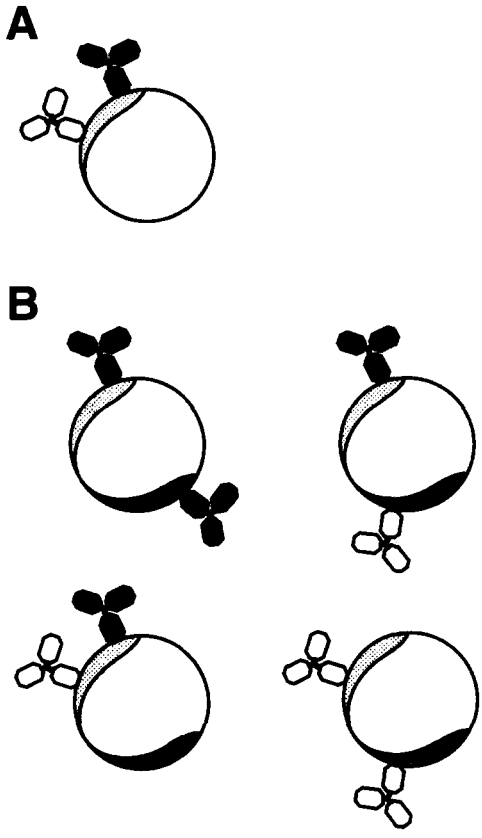

Fig. 3. Panel A shows a single chylomicron remnant particle with a single apoB-48 on its surface binding two different monoclonal antibodies, and shown in black and white. Panel B shows four images of a chylomicron remnant imagined to possess two apoB-48 molecules on its surface, binding two monoclonal antibodies in the four possible combinations.

DISCUSSION

The data presented here strongly suggest that no more than a single apoB-48 resides on the surface of the chylomicron remnant, and that the small number of particles showing two bound antibodies when a single monoclonal was added, or three bound antibodies when two different monoclonals were added, could be accounted for by random superposition of antibody molecules at the periphery of remnant particles.

The pattern of the binding of pairs of antibodies to the chylomicron remnants exhibits apparent cooperativity, as will be demonstrated from the data of Table 2. From the second and third columns of Table 2, the probability that a remnant will bind 2D8 may be estimated as 0.31, while the probability that it will bind B4 may be estimated as 0.38. If the binding of the first antibody were independent of the binding of the second, then the theoretical probability of a single remnant binding both 2D8 and B4, when these antibodies were mixed together with remnants under otherwise identical conditions, is given by the product of the independent probabilities of 0.12. But the actual probability may be estimated from the experimental data listed in the fourth column of Table 2 and is 0.36, which is almost three times the theoretical value for independent binding. Thus, the binding appears to be cooperative, so that if 2D8 were bound, then B4 was much more likely to be bound to the same remnant. Inspection of

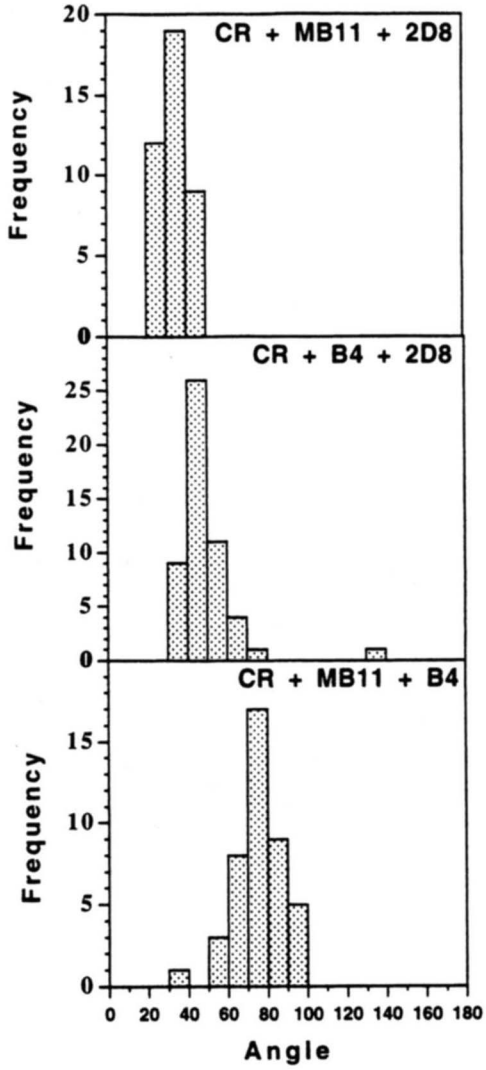

Fig. 4. Distributions of angles measured between pairs of monoclonal antibodies. Pairs of monoclonal antibodies (MB11 + 2D8, B4 and 2D8, and MB11 + B4) were mixed with chylomicron remnants and the angle between the two antibodies was determined.

the data of Table 3 leads to a similar conclusion; the binding of pairs of monoclonals was not independent but highly cooperative. Moreover, the degree of cooperativity was similar for the three pairs of the four monoclonals studied, although the distances between their epitopes varied greatly on the primary sequence of apoB-48, as shown in Table 1.

One possible explanation for the cooperativity observed in this experiment is that there are two populations of particles, one of which is competent to bind monoclonal antibodies (either one or two) and a second that is less able to bind any monoclonal antibody.

An alternative explanation for the cooperativity involves the properties of negative stain. Only molecules

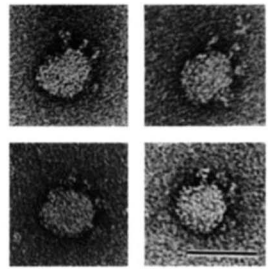

Fig. 5. Chylomicron remnant particles binding all four monoclonal antibodies simultaneously. Chylomicron remnants were mixed with a 3- to 4-fold excess of antibodies MB19, MB11, 2D8, and B4 prior to spreading. After 60 min, the preparations were diluted 50-fold and applied to the grid. Of 65 particles examined, 20 showed 4 monoclonals binding simultaneously, from which these were selected.

that displace stain are visible; therefore, an antibody that binds on top of or beneath an LDL will not be observed. The only antibodies that will be seen are those that bind to the edge of the LDL and extend out into the stain, displacing the stain. Therefore, this experiment may indicate that 36% of the time, the apoB-48 is peripherally arranged on the LDL so that all of the antibodies bound to it will be observed.

Regardless of which of these two explanations is correct, we may conclude that these data are compatible with the presence of a single apoB-48 on the chylomicron remnant but not with two apoBs. This conclusion is also consistent with the sharp angular distributions mapped for pairs of monoclonal antibodies, and consistent with simultaneous binding of four different monoclonal antibodies.

Because the B-apoproteins appear to be non-transferable among lipoprotein particles, it may be inferred that nascent chylomicrons, though of much larger diameter than the chylomicron remnants, also contain only one copy of apoB-48 at the time of secretion. One caution must be added, however. If the chylomicrons split into smaller particles during their metabolism, or if chylomicrons with two apoB-48 molecules were rapidly removed from circulation, it is still possible that the original particles could have more than a single apoB-48.

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