Differentiation of Intrahepatic Membrane-Bound and Secretory Apolipoprotein B by Monoclonal Antibodies: Membrane-Bound Apolipoprotein B Is More Glycosylated†

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ABSTRACT: Most apolipoprotein B (apoB) in rat hepatocytes membrane is membrane-bound. The purpose of this study was to determine whether differences existed between membrane-bound and plasma apolipoprotein B, which could be detected using monoclonal antibodies. Detergent-solubilized microsomal membrane-bound apoB was probed with two previously characterized monoclonal antibodies (LRB 200, LRB 220) and compared to a monospecific polyclonal antibody. LRB 200 (capable of binding 71% of plasma apoB) was able to recognize less than 20% of microsomal apoB compared to LRB 220 (a pan-apoB monoclonal antibody capable of binding 100% plasma apoB). To test the hypothesis that the immunologic difference detected by the monoclonal antibodies was due to increased glycosylation of the membrane-bound apolipoprotein B, plasma lipoproteins were incubated with neuraminidase. A progressive increase was found in antibody binding by LRB 200 but not by LRB 220 or the polyclonal antibodies. Inhibition of N-glycosylation by tunicamycin also increased the binding of monoclonal antibody LRB 200 to hepatocyte apoB. Inhibition of trimming of N-linked sugar by incubating hepatocytes with the inhibitors of glucosidase I and mannosidase I eliminated antibody binding by LRB 200 but not by LRB 220 or the polyclonal antibody. When N-linked sugars were removed by peptide: N-glycosidase F, antibody binding by monoclonal antibody LRB 200 was increased. Double-labeling experiments using ^{3}H-mannose and ^{35}S-methionine showed that cellular apoB contained twice the amount of mannose as medium apoB. These data suggest that membrane-bound apoB is more glycosylated than plasma lipoprotein apoB.

Apolipoprotein B (apoB)[1] is the major protein in very low density lipoproteins (VLDL) and low density lipoproteins (LDL) and is necessary for the secretion of VLDL and chylomicrons. Absence of apoB, as in patients with abetalipoproteinemia, results in little or no VLDL in the circulation (Salt et al., 1960). The two major forms of apoB, $apoB_H$ (apoB-100, 550 kDa) and $apoB_L$ (apoB-48, 264 kDa), are derived from a single gene product. The mRNA responsible for translation of $apoB_L$ undergoes a "C/U RNA" editing, resulting in a premature stop codon in the middle of the mRNA coding for translation of $apoB_H$ (Chen et al., 1987; Powell et al., 1987). In humans, the liver is the major site of $apoB_H$ secretion, while the intestine is the site of $apoB_L$ secretion (Glickman et al., 1986). In the rat, both $apoB_H$ and $apoB_L$ are secreted by the liver (Elovson et al., 1981).

Most of the apoB found in the liver exists in the form of membrane-associated apoB (Bostrom et al., 1986; Borchardt & Davis, 1987; Wong & Pino, 1987). The nature of this membrane-bound apoB is unknown. Some membrane-bound apoB is degraded intracellularly without ever being secreted (Borchardt & Davis, 1987). There is some recent evidence, using a partial apoB (apoB-15), that most apoB-15 is located on the cytoplasmic side of the endoplasmic reticulum (Chuck et al., 1990). Furthermore, apoB-15 has been shown to be associated with the endoplasmic reticulum membrane by virtue of a specific sequence of 33 amino acids (Chuck & Lingappa, 1992). Other investigators (Powell et al., 1991) have confirmed that a small fragment of apoB (apoB-17) is associated with endoplasmic reticulum, but there is no delayed translocation. In either case, apoB is found to be membrane-associated.

The purpose of this study was to determine whether differences between membrane-bound apoB and lipoprotein apoB could be detected using immunologic techniques, and to characterize the nature of this difference. Results show differences between membrane-bound and secreted apoB when monoclonal antibodies are used. This is most likely due to membrane-bound apoB being more glycosylated than plasma apoB. Finally, evidence is provided to show that cellular apoB is more glycosylated than secreted apoB.

MATERIALS AND METHODS

Materials. Chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated; tissue culture reagents were purchased from Gibco/BRL (Grand Island, NY); Falcon Primaria 60-mm culture plates were purchased from Baxter Scientific (Harahan, LA). Albino male rats were purchased from Holtzman Co. (Madison, WI); neuraminidase, peptide: N-glycosidase F (N-glycanase), endo-α-*N*-acetylgalactosaminidase (*O*-glycanase), castanospermine, and 1-deoxymannojirimycin were purchased from Genzyme (Cambridge, MA), and type I rat-tail collagen was purchased from Collaborative Laboratories (Bedford, MA). Monoclonal antibodies LRB 200 and LRB 220 have been described previously (Wong & Gadams, 1987). LRB 220 is a pan-apoB antibody capable of binding 100% ± 10.9% plasma apoB and LRB 200 is capable of binding 71% ± 1.3% plasma apoB. The polyclonal goat anti-rat apoB antibody also has been described (Wong &

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[1] Abbreviations: ELISA, enzyme-linked immunosorbent assay; apo, apolipoprotein; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; LDL, low density lipoproteins; VLDL, very low density lipoproteins; *N*-glycanase, peptide: *N*-glycosidase F.

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Pino, 1987) and is the same antibody used for ELISA of rat apoB. No differences were found in affinity for $apoB_H$ or $apoB_L$. D-[2-^{3}H]mannose and ^{35}S-methionine were obtained from Amersham (Arlington Heights, IL).

Preparation of Microsomal Membranes. Cell fractionation was as described (Wong & Pino, 1987) with the exception that the sucrose solution was buffered (10 mM imidazole, pH 7.0) and contained a protease inhibitor mixture (1 mM iodoacetamide, 1 mM phenylmethanesulfonyl fluoride, 11.1 trypsin inhibition units (TIU)/L aprotinin, 1 mg/L leucyl-leucyl-leucine, 10 mg/L leupeptin, 0.198 g/L, 1,10 phenanthroline, and 1 mg/L chymostatin). Microsomes were used immediately after isolation to prepare microsomal membranes using the sodium carbonate method (Fujiki et al., 1982). Protease inhibitors were added to the membrane pellet after resuspension buffer had been added (1:10 v/v). Failure to include the protease inhibitor mixture resulted in significant proteolytic degradation of apoB.

Hepatocyte Preparation. Hepatocytes were prepared according to the method of Williams (1977) with minor modifications. Rat liver was cannulated via the portal vein and flushed at 7 mL/min with Hank's balanced salt solution without calcium or magnesium. During the flush, another cannula was inserted into the inferior vena cava above the diaphragm. After 100–150 mL of Hank's solution was passed through the system, a recirculating system was established by tying off the inferior vena cava below the kidney, and perfusion with Williams medium E was begun. Perfusion medium contained 0.05 g/100 mL collagenase (Sigma Type I) and was gassed with oxygen/carbon dioxide 95%/5%. Perfusion was continued for 25–30 min at a 30 mL/min flow rate. After perfusion, the liver was minced, mixed with perfusion medium (50 mL), and incubated in a shaking water bath for 15 min at 30 °C. Following incubation, the tissue was filtered through nylon mesh (boiled panty hose) and washed once with Williams medium E. Hepatocytes were then resuspended in 30 mL of Williams medium E, diluted into 20 mL of 90% Percoll and 10% 10X Hank's balanced salt (v/v), and spun at 500*g* for 20 min. The pellet was washed another three times with Williams medium E before being resuspended in Williams medium E containing 10% fetal bovine serum. Approximately 2 000 000 cells were plated onto plates that were previously coated with rat-tail collagen (120 μg/dish). Plates were incubated for 2 h for cell attachment before the medium was removed. A totally defined artificial medium was then used (Lanford et al., 1989), and cells were incubated for an additional 16 h.

For experiments with tunicamycin, hepatocytes were washed three times with Dulbecco's phosphate-buffered saline and incubated with or without tunicamycin (2 μg/dish in 20 μL of ethanol) for 2 h. Plates were washed, cells removed with collagenase, and cells were further washed with Dulbecco's phosphate-buffered saline. The pellets were then dissolved in 0.25 mL of 0.05 M Tris buffer (pH 7.0) containing 0.1% SDS and the protease inhibitor mixture.

For experiments with glycosidase inhibitors, hepatocytes were washed as above and incubated with or without glycosidase inhibitors (400 μg each of castanospermine and 1-deoxymannojirimycin per plate) for 4 h. Washing and cell solubilization were the same as described above.

For double-label experiments, cells were incubated with 100 μCi of ^{3}H-mannose and 200 μCi of ^{35}S-methionine per plate in methionine-free Dulbecco's minimum Eagle's medium for 0–4 h. At each time point, the cells and medium were collected. ApoB was immunoprecipitated from the respective solubilized cells and medium and was separated on SDS–PAGE. Gel bands were excised and counted.

Glycosidase Incubations. Incubations using N-glycanase followed the recommended protocol from the manufacturer. Briefly, 10 μL (20 μg of protein) of resolubilized pellet (in 0.05 M sodium phosphate, pH 7.7, containing 0.5% SDS and the protease inhibitor mixture) was boiled for 5 min. To this mixture, 5 μL of 7.5% NP-40 and 0.3 unit of N-glycanase were added. The mixture was incubated overnight at 37 °C. Following incubation, 50 μL of SDS gel sample buffer (0.05 M Tris-HCl, pH 6.8, containing 4% SDS, 400 mM dithiothreitol, 10% glycerol, and 0.002% *m*-cresol purple) was added to the sample, and the sample was boiled for 5 min before application to a 3–20% gradient SDS gel.

The incubations using O-glycanase (endo-α-*N*-acetylgalactosaminidase) were similar to those of N-glycanase, except the buffer used was 20 mM sodium phosphate titrated to pH 6.0 with acetic acid. The samples contained 400 μg of protein and 13 milliunits of enzyme.

The incubations with neuraminidase used 100 μg of apoB-containing lipoproteins ($d < 1.063$ g/mL) and 80 milliunits of enzyme in a 0.02 M citrate buffer, pH 5.5, at 37 °C for varying times. At the end of a prescribed time, the sample was boiled with 50 μL of SDS gel sample buffer and applied to a 3–20% gradient SDS gel.

Labeling and Immunoblotting. Rabbit anti-goat antibody was iodinated using the chloramine-T method (Hunter & Greenwood, 1962). Specific activity was (2.6–3.3) × 10^6 cpm/μg of protein for the rabbit antibody and (2.4–3.1) × 10^6 cpm/μg of protein for the rabbit anti-mouse antibody.

After SDS electrophoresis, gels were transferred to a blotting apparatus (Hoefer, San Franscisco, CA), and proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH) as described previously (Wong et al., 1985). Transfer took place for 16 h at 30 V. Following transfer, nitrocellulose membranes were quenched with 5% defatted milk powder dissolved in 0.01 M sodium phosphate buffer, pH 7.4, containing 0.05% Tween 20 and 0.145 M NaCl (PBST buffer). Reaction with the primary antibody occurred when it was diluted with 5% defatted milk and PBST buffer and then incubated at room temperature for 4 h. The strips were washed three times for 10 min each and incubated with 10^6 cpm of secondary antibody, also diluted in 5% defatted milk and PBST buffer, for 4 h at room temperature. The strips were again washed three times with PBST before drying. Quantification of radioactive bands was done using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Preliminary experiments have shown that transfer of apoB was complete after 16 h. Further, the transfer appeared to be quantitative. Lipoproteins with various $apoB_H$ to $apoB_L$ ratios (quantified by dye elution) were completely transferred to nitrocellulose membrane and there was no residual apoB in the gel. Radioactivity ratios, when quantified by the PhosphorImager, were within experimental error of those determined by dye elution.

Other Analytic Techniques. Protein concentrations were measured using the method of Lowry et al. (1951).

RESULTS

In a previous study, we characterized several polyclonal and monoclonal antibodies to rat apoB (Wong & Gadams, 1987). Monoclonal antibody LRB 220 was capable of binding 100% ± 10.9% plasma apoB, and LRB 200 was capable of binding 71% ± 1.3% plasma apoB. None of the antibodies distinguished $apoB_H$ from $apoB_L$. We wanted to determine if microsomal membrane-bound apoB was different from plasma apoB using immunologic techniques.

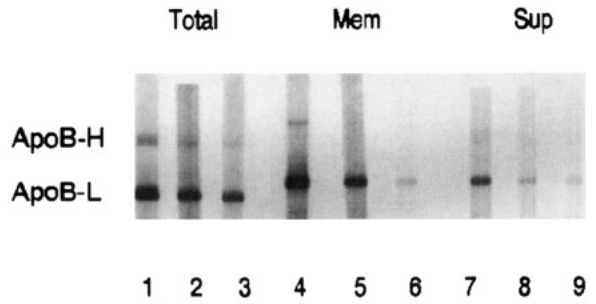

FIGURE 1: Apolipoprotein B in liver microsomes (lanes 1–3), microsomal membranes (lanes 4–6), and supernatant (lanes 7–9) as probed with polyclonal antibody (lanes 1, 4, and 7), monoclonal antibody LRB 220 (lanes 2, 5, and 8), and monoclonal antibody LRB 200 (lanes 3, 6, and 9). Note both $apoB_H$ and $apoB_L$ were present in all three fractions. Monoclonal antibodies were able to detect mostly $apoB_L$.

Table 1: Percent $ApoB_H$ in Total Microsomes, Microsomal Membranes, and Supernatant[a]

	% $apoB_H$		
antibody	total microsome	membrane	supernatant
polyclonal	11.3	11.1	23.8
LRB 220	22.1	21.0	27.9
LRB 200	35.2	39.2	40.0

[a] Total microsomes were prepared as described in the Materials and Methods section. Total microsomes were subjected to sodium carbonate treatment to separate the membrane and supernatant, then applied to an SDS gel and electrotransferred before being probed with polyclonal and monoclonal antibodies. Results represent the mean of duplicate studies. Percent $apoB_L$ binding is percent $apoB_H$ shown above subtracted from 100%.

Proteins from total microsomes, microsomal membranes, and the supernatant from microsomal membrane isolation were separated by SDS–PAGE, electroblotted, and then probed with a polyclonal antibody and monoclonal antibodies LRB 220 and LRB 200. Distribution of $apoB_H$ and $apoB_L$ in microsomes, microsomal membranes and supernatant from microsomes, when probed with either the polyclonal or the two monoclonal antibodies is shown in Figure 1. These antibodies were able to bind both $apoB_H$ and $apoB_L$ (Figure 1, lanes 1–3). The polyclonal antibody bound more to the three fractions than did the monoclonal antibodies (Figure 1, compare lanes 1, 4, and 7 with the others). If we express the binding of the individual antibodies to $apoB_H$ and $apoB_L$ as 100% regardless of the absolute counts, then we could look at relative antibody binding to $apoB_H$ or $apoB_L$. For example, the binding of the polyclonal antibody to $apoB_H$ in total microsomes is 11.3% (Table 1, row 1, column 1), then the binding of polyclonal antibody to $apoB_L$ will be 88.7%. The percent binding of antibodies to $apoB_H$, shown in Table 1, suggests both monoclonal and polyclonal antibodies bound more to $apoB_L$ in all fractions. All three antibodies recognized more $apoB_H$ in the supernatant fraction of the microsomes compared to membrane fractions (compare columns 2 and 3 of Table 1). Because 80–90% of microsomal apoB was membrane-bound (Wong & Pino, 1987), the radioactivity that was associated with antibody binding to $apoB_H$ was similar for total microsomes and microsomal membranes (columns 1 and 2 of Table 1).

The amount of radioactivity resulting from antibody binding to Western blots of apoB is shown in Figure 2. Polyclonal antibodies recognized membrane-bound apoBs twice as much as supernatant apoB (Figure 2, bottom). In contrast, LRB 220 (monoclonal pan-apoB) recognized membrane-bound apoB nine times more than the supernatant apoB. LRB 200 recognized very little membrane-bound apoB (Figure 2, top).

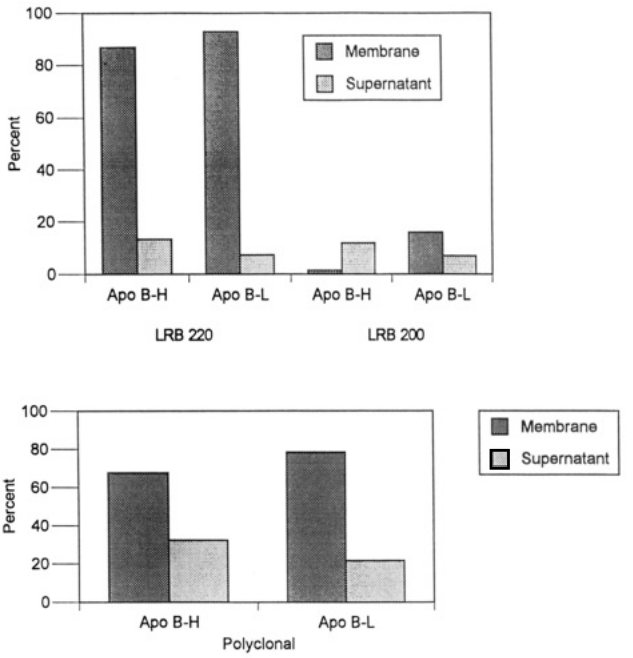

FIGURE 2: (a) (Top) Quantitative distribution of radioactive counts of $apoB_H$ and $apoB_L$ in membrane and supernatant. Total $apoB_H$ in the membrane plus supernatant as probed with monoclonal antibody LRB 220 is expressed as 100% (total counts 3417 cpm). Similarly, the total $apoB_L$ of membrane plus supernatant as probed with monoclonal antibody LRB 220 is expressed as 100% (16 013 cpm). The ratio of $apoB_H$ to $apoB_L$ is 0.21. Note that monoclonal antibody LRB 220 recognized membrane-bound apoB approximately 9 times more than supernatant apoB. Recognition of membrane-bound $apoB_H$ by monoclonal antibody LRB 200 was very low. Overall, recognition of apoB by LRB 200 was low. This is a representative experiment and is intended to compare the relative amount of the two monoclonal antibodies' binding to membrane and supernatant fractions. (b) (Bottom) Quantitative distribution of $apoB_H$ and $apoB_L$ in membrane and supernatant as probed with polyclonal antibody. Polyclonal antibody recognized more supernatant apoB than was detected by monoclonal antibody LRB 220. This is a representative experiment and is intended as an additional control.

Despite added protease inhibitors, breaking open the microsomes inevitably led to a decrease in antibody binding to $apoB_H$ (Figure 1, lanes 2, 3, 5, 6, 8, and 9).

On the basis of results shown above, we hypothesized that binding of monoclonal antibody LRB 200 to microsomal membrane-bound apoB might be sterically inhibited by oligosaccharides not yet trimmed prior to apoB secretion. If this hypothesis is correct, then removal of oligosaccharides from plasma apoB should increase binding of LRB 200 to apoB from 70% to close to 100%. To test this hypothesis, plasma lipoproteins ($d < 1.063$ g/mL) were subjected to neuraminidase treatment to remove the terminal sialic acid. The lipoproteins were then separated by SDS–PAGE, electroblotted, and probed with the different antibodies. Antibody binding of both $apoB_H$ (Figure 3, top) and $apoB_L$ (Figure 3, bottom) increased with time using monoclonal antibody LRB 200. No increase occurred in antibody binding when neuraminidase-treated lipoproteins were probed with LRB 220 or with the polyclonal antibody. Since both O-linked and N-linked oligosaccharides are sialated, we could not deduce from the data whether O-linked or N-linked oligosaccharides were responsible for inhibition of antibody binding (Figure 3).

To further characterize the nature of oligosaccharide-inhibited epitope on apoB, hepatocytes were incubated with or without tunicamycin. Hepatocytes were chosen because we could not use tunicamycin on whole animals. Tunicamycin inhibits the transfer of dolichol-oligosaccharide to asparagine

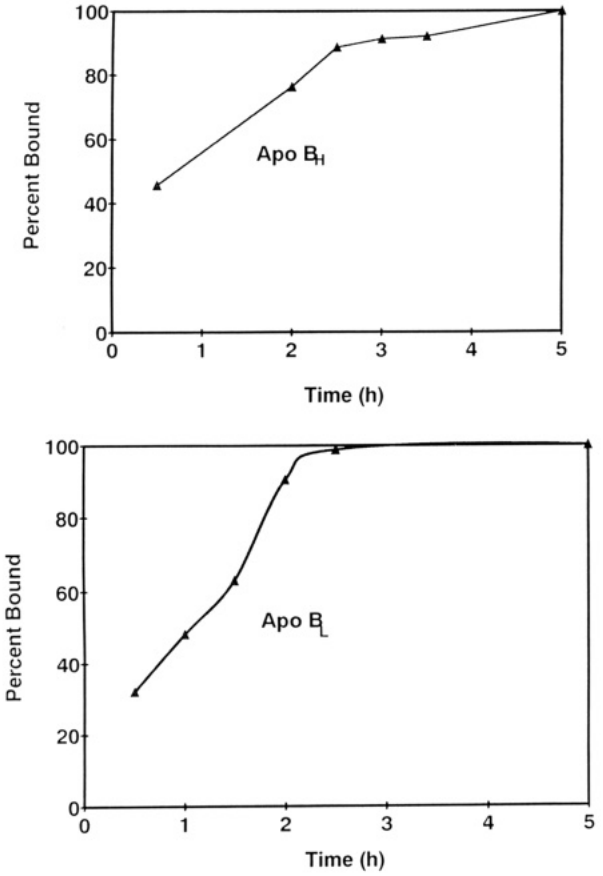

FIGURE 3: (a) (Top) Effect of neuraminidase on antibody binding of $apoB_H$. Rat VLDL was incubated with neuraminidase for varying times. After incubation, VLDL was subjected to SDS–PAGE and probed. No increase was noted in antibody binding with the polyclonal antibody or LRB 220. In contrast, a linear increase was found in antibody binding using LRB 200. The points are means of three incubations. (b) (Bottom) Effect of neuraminidase on antibody binding of $apoB_L$. Rat VLDL was incubated with neuraminidase for varying times. After incubation, VLDL was subjected to SDS–PAGE and probed. No increase was noted in antibody binding with the polyclonal antibody or LRB 220. In contrast, a linear increase (up to 2 h) was present using LRB 200. The points are means of three incubations.

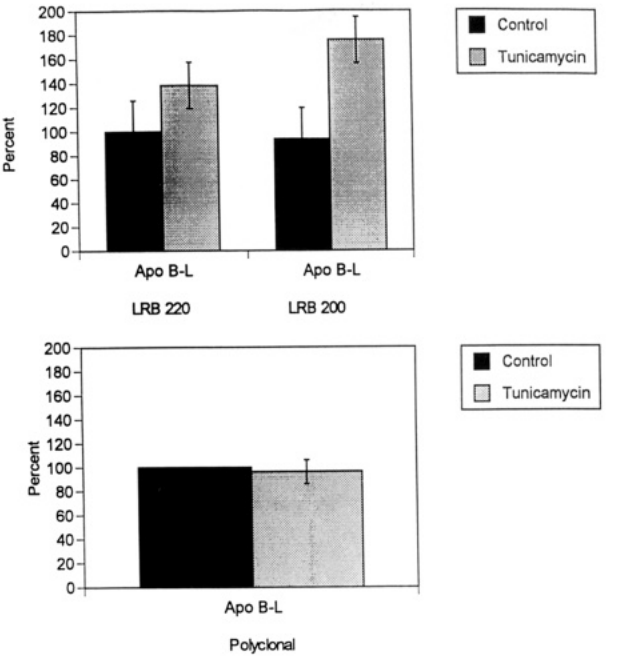

FIGURE 4: (a) (Top) Effect of tunicamycin on antibody binding of apoB. Hepatocytes were incubated with tunicamycin for 2 h before being harvested, subjected to SDS–PAGE, and probed. Equal amounts of apoB (as determined by ELISA) were applied to each gel. Tunicamycin increased LRB 200 antibody binding to apoB. Increase in LRB 220 antibody binding to apoB was not statistically significant (Student's t test). The results, expressed as percent of control LRB 220 binding, are the mean and standard deviation of four experiments. (b) (Bottom) Effect of tunicamycin on apoB as probed by polyclonal antibody to apoB. This is shown in comparison to the data shown in the top panel.

on proteins (first step of N-glycosylation). If binding by LRB 200 is affected by N-glycosylation, removal of all N-linked oligosaccharides from apoB should increase antibody binding. If binding by LRB 200 is affected by O-glycosylation, or if the epitope is on O-linked oligosaccharides, removal of N-linked oligosaccharides should not influence binding of LRB 200 to apoB. Alternately, if LRB 200 binds to N-linked oligosaccharides on apoB, removal of all N-linked oligosaccharides should result in no binding of LRB 200 antibody to apoB.

Hepatocytes were incubated with tunicamycin for 2 h. The cells were lysed, and their contents (equal amount of apoB) were subjected to reducing SDS-PAGE and electrotransferred onto nitrocellulose membrane. Nitrocellulose membranes were probed for apoB with each of three antibodies. Binding by polyclonal antibody to apoB was equal in control or tunicamycin-treated hepatocytes (Figure 4, bottom). In contrast, there was a significant increase in binding by LRB 200 to apoB of tunicamycin-treated cells (Figure 4, top). Binding of LRB 220 to apoB in control and treated hepatocytes was not significantly different. These findings are consistent with the suggestion that the epitope on apoB to which LRB 200 binds may be near an N-glycosylation site.

To further test our hypothesis, we inhibited sugar trimming in situ. After dolichol-oligosaccharide was inserted into the proteins, the oligosaccharide tree was usually trimmed by glycosidases (glucosidase and mannosidase). If trimming was inhibited, the oligosaccharide tree on apoB will be much larger than normal. Thus, the binding of monoclonal antibody to apoB should be much less or even totally inhibited. Hepatocyte glucosidase I was inhibited with castanospermine, and golgi α-mannosidase I was inhibited with 1-deoxymannojirimycin. Cells were subjected to reducing SDS–PAGE, electroblotted, and probed with the three antibodies. Results showed that inhibition of deglycosylation abolished antibody binding by LRB 200 monoclonal antibody (Figure 5). No significant decrease was present in binding to apoB by LRB 220.

If microsomal membrane-bound apoB is more glycosylated, then removal of N-linked oligosaccharides from membrane-bound apoB also should increase binding of LRB 200 to apoB. Microsomal membranes were subjected to N-glycanase treatment that removed all N-linked oligosaccharides from glycoproteins. After this treatment, microsomal membranes were subjected to reducing SDS–PAGE followed by electrotransfer. Nitrocellulose membranes were probed with the three antibodies to apoB. $ApoB_H$ was lost during incubation despite addition of the protease inhibitor mixture, regardless of whether N-glycanase was present or not. Only $apoB_L$ was evident. Results showed that N-glycanase treatment had no effect on the ability of the polyclonal antibody or monoclonal antibody LRB 220 to bind $apoB_L$. With monoclonal antibody LRB 200, a doubling of antibody binding occurred (Figure 6). To remove all O-linked oligosaccharides, microsomal membranes were incubated with O-glycanase. Again, only $apoB_L$ was evident after the incubation. No increase in binding was observed by monoclonal antibody LRB 200 or LRB 220 or the polyclonal antibody ($n = 4$, SD ± 20%, data not shown).

Direct evidence that membrane-bound apoB is more glycosylated was obtained when hepatocytes were labeled with

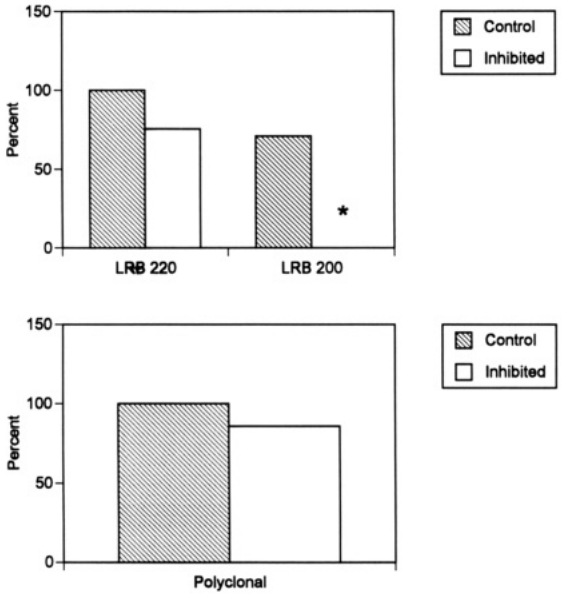

FIGURE 5: (a) (Top) Effect of glucosidase and mannosidase inhibitors on antibody binding of apoB. Hepatocytes were incubated with castanospermine and 1-deoxymannojirimycin for 2 h before being harvested, subjected to SDS–PAGE, and probed. Equal amounts of apoB (as determined by ELISA) were applied to each gel. There was a slight decrease in antibody binding using LRB 220. However, there was complete inhibition of binding by LRB 200. Results are expressed as percent of LRB 220 antibody binding to apoB in the control sample. The values are means of four experiments. The values were compared using Student's t test. The inhibition in binding of LRB 200 in the glycosidase inhibited sample was significant at 0.001 level. (b) (Bottom) Effect of glucosidase and mannosidase inhibitors on polyclonal antibody binding to apoB in hepatocytes.

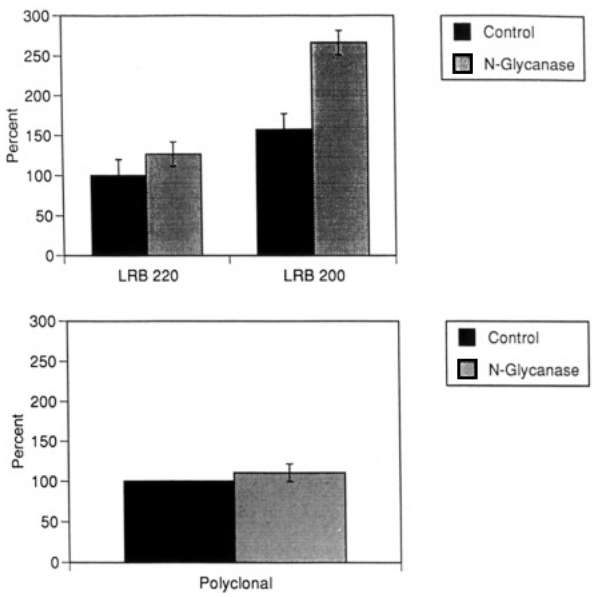

FIGURE 6: (a) (Top) Effect of N-glycanase on microsomal membrane-bound apoB. Microsomal membranes were prepared using the sodium carbonate treatment and then solubilized and incubated with or without N-glycanase for 16 h at 37 °C. The mixture was subjected to SDS–PAGE, electrotransferred, and probed with the various antibodies. Results are expressed as percent of LRB 220 binding to control membrane and are the mean and SD of four separate experiments. While there were no significant differences in binding between control and N-glycanase-treated microsomes using LRB 220, there was a significant increase in binding ($p < 0.05$ level) using LRB 200. (b) (Bottom) Effect of N-glycanase-treated microsomes on $apoB_L$ recognition by polyclonal antibody. Equal amounts of microsomal apoB were applied to the gel in all cases.

[2-^{3}H]mannose and ^{35}S-methionine from 0 to 4 h. ApoB in the medium and within hepatocytes was immunoprecipitated

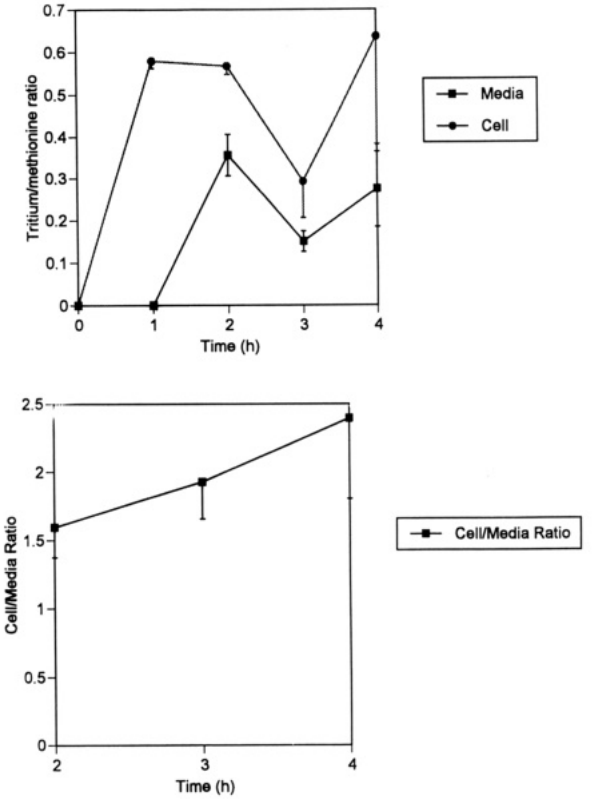

FIGURE 7: (a) (Top) Hepatocyte apoB is more mannosylated. Hepatocytes were labeled with tritiated mannose and ^{35}S-methionine from 0 to 4 h. At the end of each hour, medium and cells were isolated and apoB was immunoprecipitated and separated on SDS–PAGE. ApoB bands were then excised and counted using a liquid scintillation counter. Values represent mean and SD of three experiments. Cell carbohydrate to protein ratios increased from 0 to 0.6 by 1 h. The medium carbohydrate to protein ratio did not become significant until 2 h after labeling. Note that the medium ratio paralleled the cell ratio and was always lower than the cell ratio. (b) (Bottom) Cellular apoB has more mannose than medium apoB. The carbohydrate to protein ratio shown in the top panel was plotted as cell to medium ratio. This showed that apoB in the cell increased from 1.60 ± 0.2 to 2.39 ± 0.59 from 2 to 4 h.

and separated by SDS–PAGE. Gel bands were excised and counted. Consistent with the immunochemical data, very little labeling of $apoB_H$ occurred. The carbohydrate to protein ratio in cells reached a plateau after 1 h and decreased after 3 h (Figure 7, top). The carbohydrate to protein ratio in the medium did not show appreciable counts until 2 h after labeling (Figure 7, top); thereafter, the ratios were parallel to those of the cells but were always lower than that of cells. The cell to medium ratio of $apoB_L$ of hepatocytes increased from 1.60 ± 0.2 at 2 h to 2.39 ± 0.59 ($n = 3$) by 4 h (Figure 7, bottom). Thus, it appears that membrane-bound apoB has twice the amount of mannose as secreted apoB.

To rule out the possibility that the observed results with N- or O-glycanase, described above, occurred as a result of the enzymes interacting nonspecifically with the monoclonal antibodies and were not specific to apoB, the experiments were repeated using human fibroblasts, which contained no rat apoB, versus rat hepatic microsomal membranes. No antibody binding occurred when fibroblasts were substituted for rat liver microsomes with either N- or O-glycanase treatment.

The nature of the disappearance of $apoB_H$ also was investigated. Plasma lipoproteins ($d < 1.063$ g/mL), detergent-solubilized microsomal membranes, and a mixture of plasma lipoproteins and detergent-solubilized microsomal membranes were either incubated on ice or at 37 °C overnight before SDS–PAGE and electrotransfer. The blots were probed

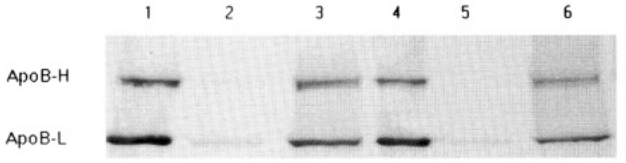

FIGURE 8: Incubation of rat plasma lipoproteins ($d < 1.063$ g/mL), hepatic microsomal membranes, and a mixture of microsomal membranes and lipoproteins at 4 and 37 °C. Lane 1 is lipoproteins incubated at 4 °C; lane 2 is microsomal membranes incubated at 4 °C; lane 3 is lipoproteins and microsomal membranes incubated at 4 °C; lane 4 is lipoproteins incubated at 37 °C; lane 5 is microsomal membranes incubated at 37 °C; and lane 6 is lipoproteins and microsomal membranes incubated at 37 °C. Incubation of lipoproteins at 37 °C reduced binding of antibody to $apoB_H$ by 16% and $apoB_L$ by 13%. Mixing lipoproteins and microsomal membranes and incubating at 4 °C (lane 3) reduced antibody binding to $apoB_H$ by 25% and $apoB_L$ by 26%. Incubation of lipoproteins and microsomal membranes at 37 °C (lane 6) reduced antibody binding to $apoB_H$ by 49% and $apoB_L$ by 52%. These findings were similar to incubation of microsomal membranes alone at 37 °C (lane 5), which reduced antibody binding to $apoB_H$ by 46% and $apoB_L$ by 48%. Most of the membrane-bound apoB appeared to be $apoB_L$ (see Figure 1, lanes 1, 4, 5, and 6); therefore, reduction of antibody binding of apoB by 50% caused $apoB_H$ to be below detectable limits.

with polyclonal antibodies. Results suggested that incubation at 37 °C overnight decreased antibody binding to apoB by 13–16% (Figure 8). Incubation overnight at 37 °C with lipoproteins suggested that $apoB_H$ did not disappear without cause. Incubation of lipoproteins with microsomal membranes at 37 °C caused a 49–52% reduction in binding. Incubation of detergent-solubilized microsomal membranes alone at 37 °C also caused a reduction in binding by 46–48%. Most likely, the disappearance of $apoB_H$ and reduction in binding of $apoB_L$ was caused by a membrane-bound protease in spite of added protease inhibitors. Experiments using lipoproteins and various proteases (trypsin, chymotrypsin, thrombin, papain, etc.) were done and resulted in complete failure of monoclonal antibodies to recognize any of the fragments (data not shown). All incubations were at 37 °C for 2 h in a 100:1 lipoprotein:enzyme ratio. The incubation with chymotrypsin and thrombin occurred at pH 8.0. The trypsin incubation occurred at pH 9.5, and the papain incubation was at pH 7.0.

DISCUSSION

Data from this study suggest that rat microsomal membrane-bound apoB contained twice the amount of mannose as plasma apoB. Monoclonal antibody LRB 200 can differentiate between membrane-bound and plasma apoB. This conclusion is based on experiments whereby N-linked oligosaccharides were removed by N-glycanase; N-linked glycosylation was inhibited by tunicamycin; N-linked oligosaccharides were prevented from being trimmed by castanospermine and 1-deoxymannojirimycin; and the terminal sialic acid of plasma apoB was removed by neuraminidase.

The question could be raised that the structure of the membrane-bound apoB may be different from that of plasma apoB. Removal of carbohydrate might expose a hitherto hidden epitope. This has been ruled out because microsomal membranes were solubilized using SDS and reduced, and the proteins were separated by SDS–PAGE and then blotted onto nitrocellulose paper. The apoB on the nitrocellulose paper was then probed with antibodies. Most, if not all, of the secondary, tertiary, or quaternary structure of apoB would have been destroyed under these conditions. Similarly, the experiments to deglycosylate apoB were all performed in the presence of ionic (SDS) and nonionic (NP-40) detergents before electrophoresis, blotting, and antibody probing (Figure 6).

Human apoB is both N-glycosylated and O-glycosylated (Taniguchi et al., 1989; Sasak et al., 1991). No data are available concerning the glycosylation of rat apoB. Most of the sugars on human apoB are biantennary. Of the 19 potential N-glycosylation sites, as predicted by amino acid sequence, 16 were found to be glycosylated (Yang et al., 1989). We are unaware of any reported O-glycosylation sites on the sequence of apoB.

Although both forms of apoB were found on the membrane and in the supernatant, antibody binding of $apoB_L$ was much stronger (90%) than that of $apoB_H$ (10%) using both monoclonal and polyclonal antibodies (Figure 1, lanes 1, 4, and 7). Following overnight incubation of membrane-bound apoB at 37 °C, $apoB_H$ inevitably disappeared, probably as a result of proteolysis associated with the microsomal membrane (Figure 8). Addition of the protease inhibitor mixture seemed to have no effect on the disappearance of $apoB_H$. One possible explanation is that the protease responsible for membrane-bound apoB degradation was not sensitive to the added inhibitors. Alternately, the protease responsible for apoB degradation may be a proprotease that was converted to an active protease during our incubation. It is tempting to speculate that this membrane-bound protease could be specific for apoB. Recently, Thrift et al. (1992) reported that [(*N*-acetylleucyl)leucyl]norleucinal (ALLN) was an effective inhibitor of intracellular apoB degradation. This particular sulfoprotease inhibitor is probably not the one responsible for degradation of apoB during our incubation because of the addition of iodoacetamide to our protease inhibitor cocktail. Another observation was that binding of apoB by polyclonal antibody was affected by temperature (Figure 8). In this process, deamidation might have a role. Another process that might have a role is lipid peroxidation (Uchida & Stadtman, 1992). The production of 4-hydroxynonenal, as a result of lipid peroxidation, which reacts with sulfhydryl groups on proteins might have profound effects on apoB conformation.

The pathway and topography of N-glycosylation have been well established (Abeijon & Hirschberg, 1992). After addition of the $Glc_3Man_9GlcNac_2$ oligosaccharide via a dolichol intermediate on the lumenal side of the endoplasmic reticulum (ER) to the protein (probably cotranslationally), the Glc_3 then undergoes deglucosidation in the ER before further demannosylation in the trans Golgi (Abeijon & Hirschberg, 1992). *N*-Acetylglucosamines are added to the trimannosyl core, followed by galactose and sialic acid. Most of the sugar addition occurs in the cis Golgi region just before protein secretion. Deglucosidation is not completely dependent on ER glucosidase I, and Golgi mannosidase also can function as a glucosidase I when ER glucosidase I is inhibited by castanospermine (Moore & Spiro, 1990). Therefore, we used both a glucosidase inhibitor (castanospermine) and a mannosidase inhibitor (1-deoxymannojirimycin) to inhibit oligosaccharide trimming. This treatment inhibited all antibody binding by LRB 200 (Figure 5). Thus, it appeared that membrane-bound apoB had already undergone some limited form of deglucosidation.

In contrast to the inhibition of deglucosidation, tunicamycin inhibited the addition of oligosaccharide to the protein at the dolichol phosphate level. Therefore, addition of tunicamycin should increase antibody binding of LRB 200. Our observations were consistent with this prediction. It should be noted that addition of tunicamycin did not inhibit synthesis and secretion of apoB (Bell-Quint et al., 1981). We were able to confirm this by performing ELISA on apo B in the medium and by immunoprecipitation of labeled apoB from the medium after cells were treated with ^{35}S-methionine and tunicamycin.

Considering the inhibition, tunicamycin and neuraminidase data together suggest that LRB 200 recognizes a peptide epitope and not an oligosaccharide epitope (Figures 3–5).

Because the two monoclonal antibodies recognized both forms of apoB in plasma, the likely epitope must be confined to the amino-terminal half of apoB. Assuming a high degree of homology between human apoB and rat apoB, we might speculate on possible sites of recognition of the monoclonal antibodies. Only five N-glycosylation sites are on the amino half of human apoB (Yang et al., 1989). Thus, it is possible to speculate that the region of LRB 200 recognition must reside close to the five N-glycosylation sites. One site (amino acids 1341–1350) contains two N-glycosylation sites and no glutamine or asparagine and could be a candidate for the LRB 200 epitope.

Chuck and Lingappa (Chuck et al., 1990; Chuck & Lingappa, 1992) proposed a mechanism for the delayed translocation of apoB. They used a partial apoB (apoB-15); therefore, it is possible that full-length apoB might contain sufficient information to cause not delayed translocation but retention in the membrane. They do not explain why approximately 80% of the intrahepatic apoB is membrane-bound (Bostrom et al., 1986, 1988; Borchardt & Davis, 1987; Wong & Pino, 1987). In any case, other investigators have disputed the delayed translocation idea (Powell et al., 1991). We postulated that membrane-bound apoB might be a storage pool for assembly of VLDL (Wong & Pino, 1987). Data from this current study provide further evidence for this suggestion. The highly mannosylated membrane-bound apoB is consistent with the idea of a newly synthesized apoB. Enzymes for N-glycosylation and sugar trimming are located lumenally (Abeijon & Hirschberg, 1992); therefore, our data also suggest that most of the membrane-bound apoB is located lumenally instead of cytoplasmically within the ER. If we consider data presented here along with that published by others, the first step in VLDL assembly appears to be synthesis of membrane-bound apoB. Oligosaccharides are probably added either cotranslationally or as apoB is translocated into the lumen. This lumenal apoB may be a storage pool of apoB. As VLDL is synthesized, some of the lumenal apoB may move through the ER to the trans Golgi, where more lipid would be added (Boren et al., 1990) and oligosaccharides trimmed. Finally, galactose and sialic acids would be added to apoB in the cis Golgi and VLDL would be secreted. Thus, as VLDL matures, apoB on the VLDL would contain less oligosaccharide than membrane-bound apoB. ApoB devoid of carbohydrates could also be secreted (Bell-Quint et al., 1981), although at a lower rate; therefore, it is unlikely that carbohydrates are necessary for proper protein folding. Thus, one might speculate that carbohydrates play a regulatory role in the secretion of apoB in VLDL. The higher carbohydrate content in the membrane-bound apoB may be a signal for apoB retention. If carbohydrates are not trimmed, VLDL synthesis may not occur. Alternately, the rate of intracellular apoB degradation might be increased by lack of carbohydrate trimming. There may be a divergence between $apoB_H$ and $apoB_L$. $ApoB_H$ could be constitutively synthesized and secreted, whereas $apoB_L$ might be the storage pool. Therefore, more $apoB_H$ would be found in the supernatant of microsomes (Figure 2). Indeed, Sparks and Sparks have presented evidence for a slower cellular pool of apoB, mostly $apoB_L$, in cultured hepatocytes (Sparks & Sparks, 1990) as opposed to a faster plasma turnover pool of $apoB_L$ (Elovson et al., 1981).

In conclusion, we have provided evidence that suggests membrane-bound apoB, mostly $apoB_L$, is more glycosylated than its plasma form.

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