Thrombin cleavage of apolipoprotein B_h of rabbit LDL: structural comparisons with human apolipoprotein B-100

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Abstract Rabbit plasma low density lipoprotein (LDL) contains one major apolipoprotein of apparent molecular weight of 320 kDa, designated apolipoprotein (apo) B_h, while another component termed $apoB_l$ of apparent molecular weight of 220 kDa is found in chylomicrons. The fragments generated by thrombin digestion of the protein moieties of rabbit and human LDL were separated by polyacrylamide gradient gel electrophoresis and compared. As in the human species, the enzyme produced limited cleavage patterns of rabbit LDL apoB. Within the first 2 h, two fragments (T_{r1} and T_{r2}, with apparent molecular weights 280,000 and 44,000, respectively) appeared. Longer incubations led to the production of two additional peptides, T_{r3} and T_{r4} (apparent molecular weights 180,000 and 96,000, respectively). Ten monoclonal antibodies, developed against rabbit LDL and designated P01 to P10, were found to react with rabbit apoB. Some also cross-reacted with human apoB. Epitope mapping, performed with these antibodies, showed that T_{r3} and T_{r4} were derived from the further degradation of T_{r1}. The rabbit is one of the most frequently used animals in atherosclerosis research. Its LDL receptor has been characterized and there exists a strain of homozygous LDL receptor-deficient rabbits referred to as WHHL rabbits. Despite this, little has been done to characterize the structure of rabbit apoB; only a short region has been sequenced and shown to be the carboxyl-terminal region, the rabbit $apoB_l$. The molecular weight of human apoB (550,000) is much larger than rabbit $apoB_h$. In both species, a primary and secondary thrombin cleavage occur, but the size of the fragments produced is very different between the two species. Identification of the thrombolytic fragments of the rabbit apoB have afforded the opportunity to compare the structures of both apoB species.—**Leroy, A., G. Castro, G. Agnani, R. Saïle, A. Barkia, and J. C. Fruchart.** Thrombin cleavage of apolipoprotein B_h of rabbit LDL: structural comparisons with human apolipoprotein B-100. *J. Lipid Res.* 1992. **33:** 889–898.

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Apolipoprotein B (apoB) is the major, nonexchangeable structural apolipoprotein of the cholesterol- and triacylglycerol-rich lipoproteins in plasma (chylomicrons, very low density lipoproteins (VLDL), intermediate (IDL), and low density lipoproteins (LDL)) (1). Mammals display at least two well-defined species of apolipoprotein B. One is a large polypeptide (apoB-100) synthesized in the liver while the other is smaller (apoB-48) and is synthesized by the intestine (2, 3). Whereas apoB-100 binds with high affinity to the LDL receptor, apoB-48 does not (4). In addition to these two proteins there are numerous reports of smaller species of apoB, which now have been shown to be proteolytic fragments of apoB.

Kane (3) has proposed a centile nomenclature for the apoB-related polypeptides, based on their apparent molecular weights on SDS-polyacrylamide gel electrophoresis, as compared to that of the large liver-derived species isolated from LDL (apoB-100). In the present work, we have adapted this nomenclature to the rabbit by using the notation of Sparks and Marsh (5). In this context the B-100 and B-48 proteins of Kane (3) are equivalent to the B_h and B_l symbols of Sparks and Marsh (in the photographs only the notation of Kane (3) is used).

The rabbit, one of the most frequently used animals in atherosclerosis research, has found particular application in studies designed to examine the relationship between hyperlipoproteinemia and the development of atherosclerosis (6). One reason for this is the fact that rabbits respond remarkably to increased cholesterol in the diet by raising their plasma cholesterol levels and developing atherosclerotic lesions (7, 8). Furthermore, there exists a strain of homozygous LDL receptor-deficient rabbits, WHHL rabbits (9). The rabbit LDL receptor has been characterized, its molecular mass estimated (128,000 kDa),

Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; apoB, apolipoprotein B; $apoB_h$, apoB of higher molecular weight; $apoB_l$, apoB of lower molecular weight; ELISA, enzyme-linked immunosorbent assay; MAb, monoclonal antibody.

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and its ability to recognize human LDL demonstrated (10). In addition, the sequence of rabbit apoE has been found (11) to share with human apoE an identical sequence between amino acids 140 and 150 which is known to be responsible for the binding of the apoE to the LDL receptor (12).

The primary structure of human apoB has been elucidated (13, 14), but little is known about the structure of rabbit apoB. Only a short segment of the rabbit protein has been sequenced (14) and shown to be the carboxyl-terminal region of rabbit B_I which shares a substantial homology with the corresponding region of human apoB-48. The present study describes the thrombolytic degradation of rabbit LDL apoB and the characterization of its cleavage products. Moreover, using monoclonal antibodies produced against rabbit apolipoprotein B, we have been able to show that other regions of rabbit apoB are homologous with human apoB.

MATERIALS AND METHODS

Animals

Male New Zealand White rabbits weighing 1.8–4.2 kg were used. Samples of plasma were obtained from animals 24 h after food withdrawal. After addition of 0.27 mM EDTA, 0.62 μM chloramphenicol, and 32.54 mM glutamine (final concentration), all plasma samples were kept at 4°C or frozen at −20°C until required.

Lipoprotein density classes

VLDL, LDL, and HDL were isolated from fresh plasma by sequential preparative ultracentrifugation (15, 16). The protein content was determined by the method of Lowry et al. (17) using BSA as standard.

Digestion of LDL B-100 with thrombin

Enzymic digests were performed as described by Cardin et al. (18) at 15°C with 100 μg of LDL B-100 protein in 0.1 ml of 10 mM Tris-HCl, 0.01% NaN_3, pH 8.0. Digestion with thrombin (Boehringer, Mannheim, RFA) was performed at an enzyme:apoB-100 protein ratio of 1:100 (w/w). Enzyme reactions were terminated by mixing the samples with 0.235 ml of buffer consisting of 10 mM Tris-HCl, 1.4% SDS, 1 mM EDTA, 8.6 mM urea, 1.4% 2-mercaptoethanol, 29% sucrose, and 0.04% bromophenol blue. In some experiments the samples were stored frozen at −20°C and subjected to electrophoresis at a later time. Gel electrophoresis experiments showed that a single freeze–thaw cycle had no effect on the degradation pattern of apoB-100.

Production of monoclonal antibodies

Male Balb/C mice were immunized intraperitoneally with 100 μg of rabbit LDL ($1.019 < d < 1.063$ g/ml isolated from animals 24 h after food withdrawal) emulsified in 300 μl of complete Freund's adjuvant (CFA). The same injection was repeated 15 days later in incomplete Freund's adjuvant (IFA). One month after the final intraperitoneal injections, the animals received a booster dose of 50 μg of LDL intravenously. Three days later, the spleens were excised.

Spleen cells were fused as described by Campbell (19) with myeloma line SP2-0 in the presence of polyethylene glycol 1450 (Kodak) containing 10% (v/v) dimethyl sulfoxide (Sigma, St. Louis, MO). The cells were distributed in microtiter plates. Hybrids were double-screened for the secretion of specific antibodies against rabbit or human lipoproteins (VLDL, LDL, and HDL) by ELISA as described by Bahr et al. (20) using microtiter plates coated with rabbit or human lipoproteins and horseradish peroxidase-conjugated rabbit anti-mouse IgG (Nordic, The Netherlands). The specificity of our antibodies was controlled by immunoblotting as described below.

Cells from positive wells were cloned twice by limiting dilution, and ascitic fluid was prepared by intraperitoneal injection of $2–4 \times 10^6$ hybridoma cells. To purify the monoclonal antibodies (IgG1), they were first concentrated by precipitation in 50% saturated ammonium sulfate at 25°C, then dialyzed against 0.1 M, phosphate buffer, pH 8, and purified by adsorption on a Protein A-Sepharose column (Pharmacia, Sweden) equilibrated in the dialysis buffer, and eluted with 0.1 M citric acid (pH 6). We also used two commercially available monoclonal antibodies: BL_3 and BL_7 (Interchim Sera-Lab, France) directed against human apoB.

Immunoglobulin class and subclass

A sample of each hybridoma supernatant was analyzed by double immunodiffusion in 1% agarose in veronal buffer as described by Ouchterlony (21), using antisera to mouse IgG (IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3), IgM, and IgA (Nordic Immunological Laboratories, Tilburg, The Netherlands).

Specificity of monoclonal antibodies

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (0.1%) (SDS-PAGE) was performed in discontinuous gels: 3% stacking gel and a 3–10% gradient separating gel (pH 8.8) for the isolation of LDL apolipoproteins according to the method of Laemmli (22). A vertical electrophoresis chamber (LKB 2100) was used, and apolipoproteins (20 μg/well) were separated over 3 h at +15°C, and 60 mA constant current per gel. The comigration of molecular weight markers (range 143,000–200,000 (BRL, USA)) was used to identify the LDL apolipoproteins on the gel.

Western blotting was performed as described by Towbin, Staehelin, and Gordon (23). Briefly, after electro-

phoretic separation, the apolipoproteins were transferred to nitrocellulose membranes (0.2 μm (LKB, Sweden)) which were then washed with 0.015 M Tris, 0.15 M NaCl, and 0.05% Tween (v/v), pH 8, and incubated overnight in the same buffer with monoclonal antibodies (20 μg/ml).

Cotitration experiment

Cotitration was carried out as described by Wong and Gadams (24), except that 1 μg/ml of rabbit lipoprotein ($1.019 < d < 1.063$ g/ml) was used for plate coating (Costar no. 3590, Cambridge, MA). To normalize the cotitration, the calculation method of Fischer and Brown (25) was used. The binding of a mixture of two monoclonal antibodies to LDL was expressed in the following way:

$$B = (A_{(a+b)}/(A_a + B_b)) \times 2$$

where B is the relative binding coefficient, $A_{(a+b)}$ is the saturation binding level of the mixture of monoclonal antibodies a and b, and A_a and A_b are the individual saturation binding levels measured for each monoclonal antibody. For pairs of monoclonal antibodies that bind to LDL, B = 2 if no competition exists between the two monoclonal antibodies, whereas B = 1 (or close to 1) if a strong competition exists between the two monoclonal antibodies. Intermediate values are obtained if there is a certain amount of competition between the two monoclonal antibodies. The assay is not suitable for comparison of monoclonal antibodies of different classes.

Radioiodination

LDL was labeled with $Na^{125}I$ using the iodine monochloride method (26). The specific activity of the ^{125}I-labeled LDL ranged from 600 to 800 cpm/ng protein in the different experiments.

Immunoprecipitation

^{125}I-labeled LDL (100 ng) was mixed with a series of dilutions of the MAb. Both the antibody and the LDL were diluted in PBS containing 1 mM EDTA and 1% BSA. The final reaction mixture volume was 200 μl. The mixture was incubated for 24 h at room temperature at which time 50 μl of precipitating second antibody (sheep anti-mouse IgG (Nordic Immunological Laboratories, Tilburg, The Netherlands)), diluted to give a slight antibody excess, was added. After a further 3 h incubation at room temperature, 1 ml of PEG 6000 (polyethylene glycol, 3% w/v) was added and the mixture was centrifuged for 15 min at 3500 rpm. The pellet was then washed once and the bound radioactivity was determined. The results are expressed as antibody-precipitable radioactivity as a percentage of trichloroacetic acid-precipitable radioactivity.

Determination of affinity constants

Competition between ^{125}I-labeled and unlabeled LDL was used to determine the affinity constant of the MAbs. The assays were performed in triplicate in Eppendorf tubes and all dilutions were made in PBS containing 1% BSA. Each sample contained 50 μl of serial dilutions of unlabeled LDL, 50 μl of ^{125}I-labeled LDL (200 ng of protein), and 100 μl of the MAb at the dilution required for approximately 50% maximum binding in the absence of competing antigen. The tubes were incubated for 18 h at room temperature and, after the addition of 100 μl of the precipitating antibody, the same procedure was followed as described above to determine the precipitable radioactivity. The affinity constants for each antibody were calculated according to the Müller equation (27):

$$K(M^{-1}) = 1/(I_t - T_t) \times (1 - 1.5\ b + 0.5\ b^2)$$

where I_t is the molar concentration of competing antigen required for 50% inhibition of ^{125}I-labeled LDL binding to the antibody; T_t is the molar concentration of ^{125}I-labeled LDL added; and b is the percentage of maximum binding of ^{125}I-labeled LDL without competitor under the conditions of the assay.

Other methods

Protein was determined by the method of Lowry et al. (17) with bovine serum albumin as standard. Gel densitometry was performed on a Zeineh soft laser scanning densitometer (Biomed Instruments, Inc., Chicago, IL). Quantitative densitometric scans were conducted in the linear range of the optical response with respect to peptide mass. The apparent molecular weights of the peptides were determined by extrapolation from a standard plot of the logarithm of the molecular weights versus the electrophoretic mobility of standard proteins. The protein standards were myosin (H-chain, 200,000), phosphorylase B (97,400), bovine serum albumin (68,000), ovalbumin (43,000), α-chymotrypsinogen (25,700), β-lactoglobulin (18,400), and lysozyme (14,300).

RESULTS

Digestion of LDL apoB_h with thrombin

In contrast to the extensive cleavage produced by trypsin or staphylococcal V_8 proteinase (28), thrombin generated only a limited number of cleavage products (**Fig. 1**).

Lane F shows rabbit LDL B_h. Lanes B–E represent rabbit LDL B_h that was digested for 1 h, 2 h, 16 h, and 50 h, respectively. Lane K shows human LDL B-100. Lanes G–J represent human LDL B-100 that was digested for 1 h, 2 h, 16 h, and 50 h, respectively.

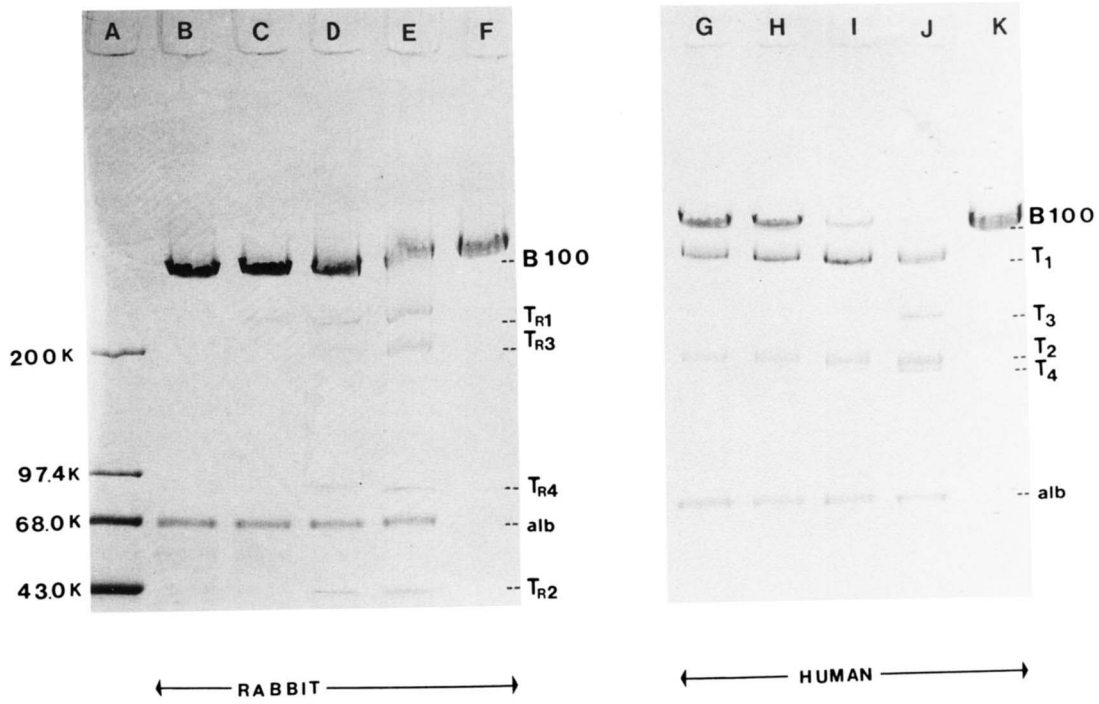

Fig. 1. SDS-polyacrylamide gradient (3–10%) gel electrophoresis of thrombin-treated rabbit and human LDL. LDL was incubated for various times with 1% human thrombin (by weight) at 15°C. The protein, 20 μg/well, was electrophoresed as described in Materials and Methods. Lane A shows standard molecular weight markers. Lanes B to E represent rabbit LDL B_h digested for 1, 2, 16, and 50 h, respectively. Lane F shows untreated rabbit LDL B_h kept for 50 h at 15°C. Lanes G to J represent human LDL B-100 that was digested for 1, 2, 16, and 50 h, respectively. Lane K shows untreated human LDL B-100 kept for 50 h at 15°C.

During the first 2 h, we found that thrombin liberated two major fragments, designated T_1 and T_2, from human LDL B-100 (lanes G and H). At times greater than 2 h, two additional cleavage products, designated T_3 and T_4, were produced in lesser amounts (lanes I and J). The apparent molecular weights of peptides T_1–T_4 were 385,000, 170,000, 238,000, and 145,000, respectively, similar to the values obtained by Cardin et al. (18).

From rabbit LDL B_h, thrombin liberated two major fragments (T_{R1} and T_{R2}, lanes B and C) during the first 2 h of incubation. Laser densitometry indicated that these peptides were produced in stoichiometric amounts. Longer incubation resulted in the formation of two additional cleavage products, designated T_{R3} and T_{R4} (lanes D and E). Based on gradient gel electrophoresis, the apparent molecular weights of peptides T_{R1}–T_{R4} were respectively: 280,000 ± 5,000, 44,000 ± 3,000, 180,000 ± 4,000 and 96,000 ± 3,500 (mean and SD of 11 different preparations). The sum of the apparent molecular weights of T_{R1} and T_{R2} (324,000 ± 8,000) and their relative stochiometries suggested that these peptides result from specific cleavage of apoB_h (320,000 ± 5,000) at a single site. The sum of the apparent molecular weights of the minor fragments, T_{R3} and T_{R4} (276,000 ± 7,500) suggested that these peptides were derived from the further degradation of T_{R1} (280,000 ± 5,000). Densitometric scans of lanes D and E of the gradient gel are represented in **Fig. 2**. Extensive thrombin degradation of rabbit LDL B_h showed that fragment T_{R1} did not increase stoichiometrically with the degradation of LDL B_h. Gel densitometry of lane E indicated a decrease in peptide T_{R1} and an increase in peptides T_{R3} and T_{R4}, while the fragment T_{R2} continued to increase stoichiometrically with the decrease of LDL B_h.

Production of hybridomas

After fusion and hypoxanthine–aminopterine–thymidine selection, there were 40 hybridomas. Of the 40, 20 were found to react strongly with rabbit LDL by the ELISA detection method. We propagated 10 of these 20 hybridomas and subcloned them by limiting dilution. The hybridomas were designated P01 to P10. Monoclonality was established by two successive limiting dilution subclonings. All the monoclonal antibodies produced had an IgG1 isotype except P09 and P08 that proved to be of the IgG_{2a} and IgM class, respectively.

Specificity of the monoclonal antibodies

All the antibodies (P01 to P10) reacted (by ELISA assay) with the two rabbit lipoprotein classes, VLDL ($d < 1.006$ g/ml), and LDL ($1.019 < d < 1.063$ g/ml) but not with HDL ($1.063 < d < 1.21$ g/ml). These results suggest that

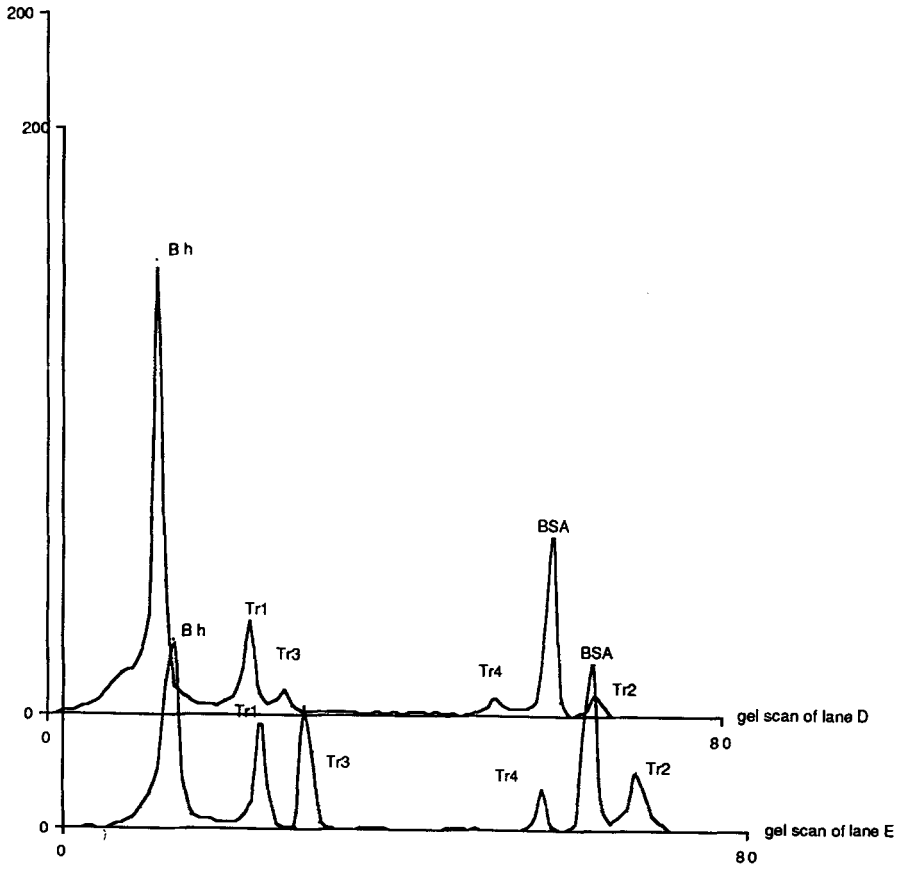

Fig. 2. Gel scan of lanes D and E of Fig. 1, representing the degradation of rabbit LDL B_h with human thrombin after 16 and 50 h of treatment, respectively.

the antibodies produced were against B apolipoproteins. Some of them: P02, P03, P04, P05, P06, P07, and P08 cross-reacted with human lipoproteins (VLDL and LDL).

Cotitration experiment

Using a solid phase assay, we determined whether purified monoclonal antibodies competed for binding to immobilized rabbit lipoprotein. P08 was not tested because it is of the IgM class. Some monoclonal antibodies were able to compete totally (P10 with P01 and P09, P04 with P03, and P05 and P03 with P05) for binding to rabbit LDL (**Table 1**). Others competed partially (e.g., P06 with P07) or poorly (e.g., P06 with P02) for binding. P10 competed partially with P02 and P06, which themselves compete poorly. These results suggest that the epitope recognized by P10 is a conformational epitope. The following antibodies: P02, P03, P06, P07, and P10 have been further characterized because they showed little competition (>1.5 in Table 1), apparently binding to distinct rabbit apoB epitopes. To this list we have added P05, because P03 did not compete with P10, while P05 competed strongly with P03 and partially with P10.

Immunological specificity of antibodies

The preceding results were substantiated by immunoblotting experiments. The selected monoclonal antibodies bound a protein with an electrophoretic mobility of $apoB_h$ (molecular mass, 320 kDa). Some of the antibodies reacted with another protein with the electrophoretic mobility of $apoB_l$ (molecular mass, 220 kDa). The antibodies did not react with other apolipoproteins on the gel as seen in **Fig. 3** (immunoblots of rabbit VLDL). These results are summarized in **Table 2** (under rabbit apoB specificity). Two monoclonal antibodies (P06, P10) appeared to recognize the $apoB_h$ (rabbit B-100) but not the $apoB_l$ (rabbit B-48) (Fig. 3, lanes D and F); the other four antibodies recognized both rabbit apoB forms (Fig. 3, lanes A, B, C, and E). We also established, by immunoblot of human VLDL with the same antibodies, that P02, P03, P05, and P07 recognized both human apoB forms (B-100 and B-48). P06 recognized only the human apoB-100 and not the apoB-48, while P10 recognized none of the human apoB forms (ELISA studies had already given negative reactions for this antibody with human VLDL and LDL).

TABLE 1. Cotitration of monoclonal anti-rabbit apoB antibodies

	Relative Binding Coefficient									
MAb	P01	P02	P03	P04	P05	P06	P07	P08	P09	P10
P01	1	1.6	1.97	2.02	1.52	1.79	1.71		1.05	1.03
P02		1	1.96	2.1	2	1.96	1.97		1.5	1.58
P03			1	1.1	1.02	2.02	1.98		2.01	2.15
P04				1	1.09	2.15	2		2.04	2.1
P05					1	2.03	2.01		1.49	1.5
P06						1	1.64		1.81	1.86
P07							1		1.69	1.73
P08										
P09									1	1.04
P10										1

Cotitration studies were performed as described in the Methods section. P02, P03, P06, P07, and P10 showed little competition (relative binding coefficient >1.5), apparently binding distinct rabbit apoB epitopes.

Estimation of maximum binding and affinity of each selected antibody

Determination of the maximum amount of ^{125}I-labeled LDL that could be bound by individual antibodies (P02, P03, P05, P06, P07, and P10), is summarized in Table 2. The maximum amount ^{125}I-labeled LDL precipitable varied from 76% to 98%. Their affinity constants for rabbit apoB epitopes on intact LDL ranged from 0.13 to 16.1 × 10^8 liter/mole (Table 2).

Location of antigenic determinants on rabbit apolipoprotein B thrombolytic fragments

Further characterization of the epitope specificity of the anti-rabbit LDL monoclonal antibodies was carried out after cleavage of rabbit LDL $apoB_h$ by thrombin. Cleavage leads to the production of four peptides. The peptides produced were separated by SDS polyacrylamide gel electrophoresis, transferred to nitrocellulose paper, and probed with the following antibodies: P02, P03, P05, P06, P07, and P10. Fig. 3 (immunoblots of thrombin-treated rabbit LDL) shows the results of one of these experiments. The results are summarized in Table 2. P03, P05, P06, P07, and P10 reacted with T_{r1} and T_{r3} as can be seen in Fig. 3 (lanes H–L) while P02 reacted with T_{r1} and T_{r4} (Fig. 3, lane G).

Comparison of the immunospecificity of two anti-human apoB monoclonal antibodies (BL_3 and BL_7) with the anti-rabbit apoB monoclonal antibodies

P02 reacts with the following rabbit peptides: T_{r1}, T_{r4}, $apoB_h$, and $apoB_l$, and with the following human pep-

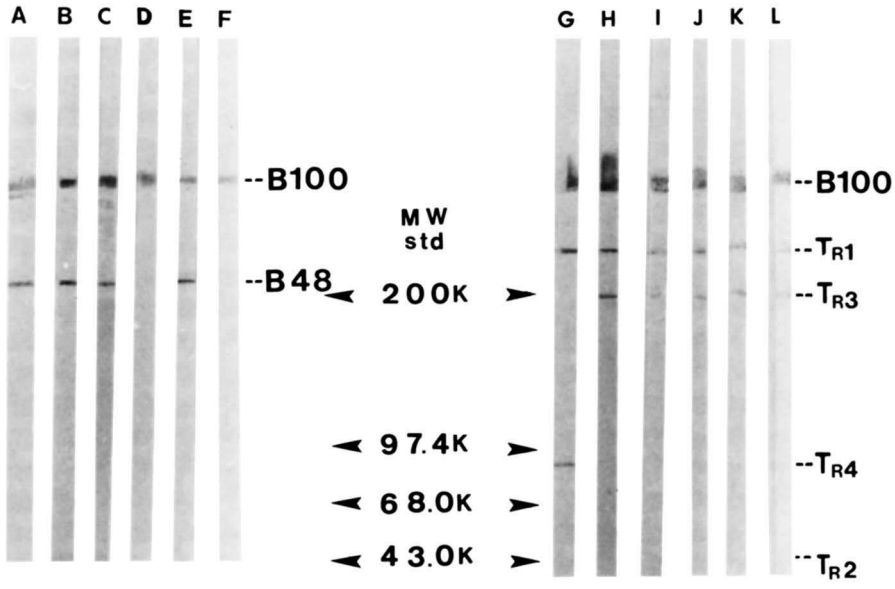

Fig. 3. Immunoblots of rabbit VLDL (lanes A–F) and thrombin-treated rabbit LDL (lanes G–L) after electrophoresis in SDS-polyacrylamide gradient (3–10%) gel. The monoclonal antibodies used were: P02, lanes A and G; P03, lanes B and H; P05, lanes C and I; P06, lanes D and J; P07, lanes E and K; P10, lanes F and L.

TABLE 2. Apolipoprotein specificity of the mouse monoclonal antibodies (MAbs) for the rabbit and human apoBs

MAb	Specificity by immunoblotting: Rabbit $ApoB_h$	Rabbit $ApoB_l$	Human ApoB-100	Human ApoB-48	Affinity Constant for Rabbit LDL	Maximum Binding for ^{125}I-Labeled Rabbit LDL	Rabbit Thrombolytic Fragments: T_{r1} 280 kDa	T_{r4} 96 kDa	T_{r3} 180 kDa
					l/mol	*% of TCA-precipitable radioactivity*			
P02	+	+	+	+	2.37 10^8	98	+	+	–
P03	+	+	+	+	2.71 10^8	78	+	–	+
P05	+	+	+	+	8.28 10^8	95	+	–	+
P06	+	–	+	–	0.20 10^8	86	+	–	+
P07	+	+	+	+	0.13 10^8	76	+	–	+
P10	+	–	–	–	16.1 10^8	94	+	–	+

Specificity was established by Western blotting, affinity constants for the binding to rabbit LDL, and determination of maximum amounts of ^{125}I-labeled rabbit LDL immunoprecipitable by each antibody, and location of antigenic determinants on rabbit apolipoprotein B thrombolytic fragments.

tides: apoB-100 and apoB-48 (Table 2). P06 reacts with the following rabbit peptides: T_{r1}, T_{r3}, $apoB_h$ but not $apoB_l$, and with the following human peptides: apoB-100 but not apoB-48 (Table 2). P03, P05, and P07 react with the following rabbit peptides: T_{r1}, T_{r3}, $apoB_h$, and $apoB_l$, and with the following human peptides: apoB-100 and apoB-48 (Table 2). We have compared the immunospecificity of the P02, P03, P05, P06, and P07 antibodies with the immunospecificity of BL_3 and BL_7 antibodies by Western blotting of the human apoB-100 thrombin fragments. The monoclonal antibodies BL_3 and BL_7 produced and partially characterized by Salmon et al. (29) were mapped using human apoB fusion proteins by Pease et al. (30). Pease et al. showed that BL_3 and BL_7 recognize residues 4342–4536 (in the T_2 thrombolytic fragment) and 2239–2376 (in the T_3 thrombolytic fragment), respectively, in human apoB.

As shown in **Fig. 4**, immunoblotting of the human apoB thrombolytic fragments with P03, P05, P06, and P07 and BL_7 (lanes B, C, D, E, and F, respectively) give a similar pattern of bands that differs from the one obtained with BL_3 (lane G) which recognizes the B-100 and T_2 fragment. Therefore, P03, P05, P06, and P07, as well as BL_7, react with the human apoB thrombin fragments T_1 and T_3 but not with the T_2 fragment. The P02 antibody was found (Fig. 4, lane A) to recognize the T_1 and T_4 human thrombin fragments.

DISCUSSION

In contrast to the extensive cleavage produced by trypsin or staphylococcal V_8 proteinase (28), thrombin generated only a limited number of cleavage products from rabbit LDL apoB similar to the results obtained for human LDL apoB (18). In the first 2 h, thrombin cleavage yielded two fragments: T_{r1} and T_{r2} with apparent molecular weights 280,000 and 44,000. More prolonged incubation liberated two additional peptides T_{r3} and T_{r4} (apparent molecular weights 180,000 and 96,000). The apparent molecular weights of T_{r1}, T_{r3}, and T_{r4} indicate that the peptides T_{r3} and T_{r4} were derived from the further degradation of T_{r1}. This suggestion has been given further support (see Table 2) by immunoblotting experiments (P03, P05, and P07 react with rabbit T_{r3}, T_{r1}, and B_l, while P02 reacts with T_{r4}, T_{r1}, and B_l). Furthermore, two other monoclonal antibodies (P06 and P10) were found to recognize the carboxyl-terminal portion of T_{r3} in that they bound to T_{r3} and did not recognize rabbit B_l. One of the two antibodies (P06) cross-reacted with human apoB-100 and not B-48, showing that this segment of rabbit apoB (carboxyl-terminal portion of T_{r3}) shares homology with human apoB. Moreover, while P07 recog-

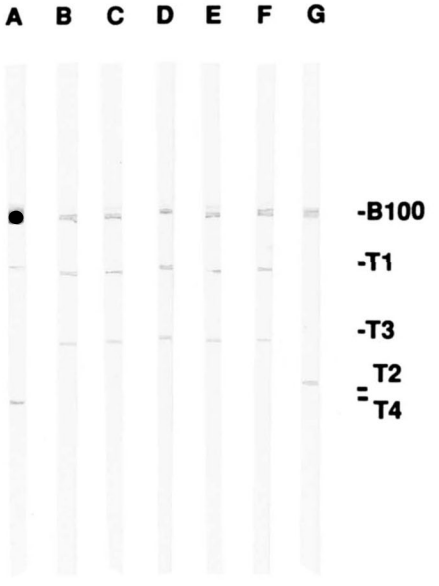

Fig. 4. Immunoblots of thrombin-treated human LDL after electrophoresis in SDS-polyacrylamide gradient (3–10%) gel. The monoclonal antibodies used were: lane A, P02; lane B, P03; lane C, P05; lane D, P06; lane E, P07; lane F, BL_7; and lane G, BL_3.

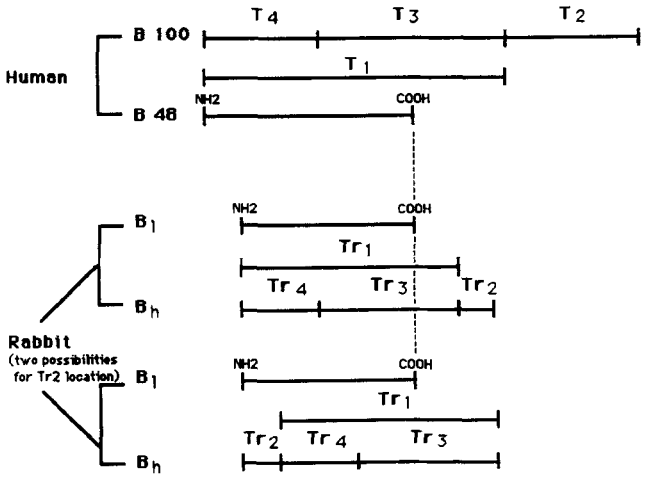

Fig. 5. Schematic representation of the thrombin cleavage sites in LDL. The major thrombin cleavage products are T_1, T_2, T_3, T_4, and T_{r1}, T_{r2}, T_{r3}, T_{r4} for human and rabbit LDL, respectively. T_1, T_2 and T_{r1}, T_{r2} are the primary peptides liberated by thrombin; T_3, T_4 and T_{r3}, T_{r4} are secondary. The vertical lines indicate the limits of the different peptides. The carboxyl-terminal region of the human B-48 and rabbit B_l have been co-aligned according to the finding of Powell et al. (14).

nized T_{r3}, T_{r1}, rabbit apoB_h, and B_l, P06 recognized T_{r3}, T_{r1}, rabbit apoB_h but not rabbit apoB_l, which establishes that the T_{r3} fragment spans the carboxyl-terminal extremity of the rabbit B_l and that the epitope recognized by P06 belongs to the carboxyl-terminal extremity of T_{r1}. As P02 recognized T_{r4}, T_{r1}, rabbit apoB_h and B_l, we localized T_{r4} at the amino-terminal extremity of the rabbit T_{r1}. Unfortunately our results do not throw light on the location of T_{r2} (either at the amino or carboxyl-terminal extremity of T_{r1}). Based on the intramolecular specificities of the murine monoclonal antibodies described herein, it would appear that the T_{r2} segment of rabbit apoB is poorly immunogenic for the mouse compared to T_{r3} and T_{r4}. A short region of rabbit apoB has been sequenced (14) and shown to be the carboxyl-terminal region of the rabbit B_l and to share a high homology with the corresponding region of the human apoB-48. Furthermore, the immunoblotting of the human apoB thrombolytic fragments with P02, P03, P05, P06, and P07 and with BL_7 (directed against the human apoB thrombin fragment T_3) show that P03, P05, P06, and P07 as well as BL_7 (Fig. 4) react with human apoB thrombin fragments T_1 and T_3 and that P02 recognizes the T_1 and T_4 human thrombin fragments. These results and data lead us to propose a schematic representation (**Fig. 5**) of the thrombin cleavage sites in rabbit LDL apoB. In this scheme, the carboxyl-terminal region of human apoB-48 and rabbit apoB_l, which have been found to be identical by Powell et al. (14), have been co-aligned. Two possibilities exist for the location of the rabbit T_{r2} fragment, but the upper representation of rabbit apoB thrombolytic fragments in Fig. 5 placing the T_{r2} fragment at the carboxyl-terminal extremity of the rabbit apoB seems most probable.

Unlike apoE, apoB has an unusually large molecular mass and when dissociated from lipid-containing particles, becomes insoluble in water (3, 31). As a result, apoB has not been amenable to the identification of domains necessary for LDL receptor interaction. Amino acid sequence comparisons across species provide a means of defining structures that are essential for function. Ebert et al. (32) have sequenced a 1.1 kb fragment of pig apoB genomic DNA, corresponding to a 363 amino acid segment that shared 72% identity with the human amino acid sequence (3132–3495). Several authors (13, 33) have proposed this segment (3132–3495) as a candidate for the mediation of human apoB binding to the LDL receptor by two regions enriched in positively charged amino acids (residues 3147–3157 and 3359–3367) located on both sides of the human T_3/T_2 thrombolytic cleavage junction (lysine-3249). Monoclonal antibodies that bind near these regions block the interaction of human apoB with the LDL receptor (13), but none of the monoclonal antibodies described here was able to decrease the interaction of rabbit LDL with the LDL receptor (data not shown).

Unfortunately, we do not yet know the primary structure of rabbit apoB, only a short region having been sequenced (14). Rabbit and human apoB undergo the same process of mRNA editing that leads to the secretion by the intestine of the light form of apoB (14). This is consistent with our results and the fact that in this region (the carboxyl-terminal extremity of B_l and B-48) several antibodies cross-react with the apoB of both species.

The present study shows that rabbit apoB can be used as an antigen to obtain monoclonal antibodies that cross-react with the T_1 thrombolytic fragments of human apoB but not against the human T_2 thrombolytic fragment. Others from our laboratory have also failed to obtain antibodies against the T_2 thrombolytic fragment using human LDL as antigen and the rat for the production of monoclonal antibodies (34). Using this technique they obtained monoclonal antibodies against the T_1, T_3, and T_4 human thrombolytic fragments. Based on the monoclonal antibodies described to date (31, 32, 35), it appears that only the immunization of mice with human LDL produces antibodies against the T_2 thrombolytic fragment.

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