Measurement of the absolute number of functioning low-density lipoprotein receptors *in vivo* using a monoclonal antibody

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MAC188 S/S is a monoclonal antibody which can be used *in vivo* to measure the absolute number of functioning low-density lipoprotein (LDL) receptors in a rabbit. The antibody binds to the extra-cellular domain of the LDL receptor and binding is not blocked by the presence of LDL. When the antibody–receptor complex is internalized, receptor recycling is inhibited for several hours. Thus, when saturating doses of MAC188 S/S are administered intravenously, the amount of antibody removed from the blood (minus non-specific removal) is determined solely by the total number of LDL receptors in an animal. In this study MAC188 S/S was used to measure the number of LDL receptors in control rabbits and in animals treated with 17 α -ethinyl oestradiol. After treatment (which caused a 47% decrease in

plasma cholesterol), receptor-mediated removal of MAC188 S/S from the blood was saturated in both groups following injection of 3.0 mg of antibody per kg body weight. Based on the amount of antibody removed via the LDL receptor at this dose, the total number of accessible LDL receptors was calculated as $(2.0 \pm 0.3) \times 10^{15}$ receptors per kg body weight in control rabbits and $(4.0 \pm 0.4) \times 10^{15}$ receptors per kg body weight in oestrogen-treated animals. The number of receptors in various organs was also determined. The monoclonal antibody approach therefore, allows accurate determination of LDL receptor numbers in animals with markedly different concentrations of circulating LDL, conditions in which the use of endogenous ligand would be subject to significant errors.

INTRODUCTION

The level of low-density lipoprotein (LDL) circulating in the blood is determined by the rate of LDL production and by the rate of LDL removal by the tissues. When the diet is low in cholesterol and triacylglycerol, the majority of LDL transport out of the plasma is mediated by the LDL receptor. The relative importance of receptor-independent transport increases, however, as animals are fed increasing amounts of cholesterol and saturated fat [1]. Clearance studies using radiolabelled LDL have enabled investigators to determine the kinetic parameters of LDL metabolism *in vivo*. Thus, the rates of receptor-mediated and receptor-independent clearance have been measured in a range of experimental animals [2,3].

In attempting to understand the factors that regulate plasma LDL concentration, it would be very useful to have a technique for the estimation of the overall activity of the LDL receptor pathway. Although the natural ligand can be used as a tracer for the LDL receptor, it has limitations as a probe for receptor quantification. First, the LDL fraction isolated from plasma is a heterogeneous spectrum of particles with different cholesterol: protein ratios and these may vary in their ability to bind to the receptor [4,5]. Secondly, serum contains other lipoproteins, very-low-density lipoprotein (VLDL) and intermediate-density lipoprotein (IDL), which also bind to the LDL receptor [6]. Measurement of the clearance of tracer LDL from the plasma will obviously be complicated by the presence of other lipoproteins, the concentration and composition of which will vary with diet, drug treatment and disease [7,8].

An alternative approach to the use of LDL as a ligand for measuring LDL receptor activity *in vivo* is the use of anti-(LDL receptor) monoclonal antibodies. So far, only two antibodies with suitable properties have been produced. The first, 9D9, was

developed by Huettinger et al. [9] and the second, MAC188, was developed in this laboratory [10]. Like 9D9, MAC188 is a monoclonal antibody which recognizes the extracellular part of the rabbit LDL receptor. It binds to the receptor with high affinity at physiological temperature and in the presence of high concentrations of the LDL [11]. These properties make MAC188 an ideal probe to quantify the receptor *in vivo*.

MAC188 has a further important property. Once it binds to receptors it is internalized and then traps the receptors inside the cell. Normal receptor recycling is inhibited and degradation of the antibody is much slower than that of the natural ligand [11]. When cells are incubated with high concentrations of MAC188, all the LDL receptors are trapped inside the cells and no LDL receptors are present at the cell surface [11].

The rapid binding of MAC188 to cell-surface receptors and subsequent trapping means that when the antibody is injected into rabbits it is quickly removed from the blood and it accumulates in the tissues [11]. It should be possible, therefore, to inject an animal with a dose of MAC188 large enough to trap all functioning LDL receptors within intracellular compartments. The amount of antibody removed from the blood during this process would be equivalent to the amount bound to the receptors and could be used to calculate the total number of functioning LDL receptors in the animal. Thus, the properties of MAC188 permit the calculation of the absolute number of receptors, whereas previously it has only been possible to determine the rate of receptor-mediated clearance, which is a composite of the rate of ligand binding and the rate of receptor recycling.

It is of course anticipated that some receptors would not be exposed to circulating antibody. These would include receptors on cells to which there is limited access to plasma proteins, as well as newly synthesized receptors not yet involved in recycling. These receptors would, however, not normally participate in

LDL transport as they would not be accessible to LDL. The total number of accessible or functioning LDL receptors is what we have previously called the LDL receptor status of an animal [12].

In a previous study, the receptor-mediated removal of injected MAC188 from the blood was only partial [11]. Even at low doses of antibody, 40–50% remained in the blood after the initial period of rapid removal. We suggested that this was due to the presence of a fraction of MAC188 that did not bind to the receptor. The hybridoma which produces MAC188 was developed by fusing spleen cells with the light-chain-secreting myeloma line, Y3. The hybridoma secretes three variants of the antibody: MAC188 S/S, a variant with two spleen light chains, MAC188 S/Y3, a hybrid antibody with one spleen light chain and one Y3 myeloma light chain and MAC188 Y3/Y3, a variant with two myeloma light chains. We considered it likely that the variant without spleen light chains, MAC188 Y3/Y3, would not bind to the receptor and would therefore not be removed from the blood following injection.

In this report we describe the isolation of the MAC188 variants and the use of the fully active MAC188 S/S variant to determine the total number of accessible LDL receptors in rabbits with differing levels of LDL receptors.

MATERIALS AND METHODS

Monoclonal antibodies used in these studies

MAC188

Rat IgG1 antibody that recognizes the rabbit LDL receptor [10,11] was used.

MAC221

Isotype-matched control for MAC188 [11]. MAC221 is directed against an irrelevant antigen (bovine placental lactogen).

Monoclonal antibody production

MAC188 and MAC221 hybridoma cells were grown in Dulbecco's modified Eagle's medium containing 5% (v/v) fetal calf serum. Initially the hybridomas were grown in stirrer bottles (Techne) but, when larger quantities of antibody were required, cells were grown in Oscillating Bubble Chambers (Cambridge Glass Blowing, Cottenham, Cambridge) [13]. Bubble chambers were inoculated with cells at $(0.5 \text{ to } 1.0) \times 10^6$ cells/ml and cultures were then maintained for up to 28 days as described by Pannell and Milstein [13].

Purifying monoclonal antibodies from hybridoma supernatant

Hybridoma cell supernatants were dialysed against 50 mM Mes, pH 6.0, and loaded on to a 4 ml NeoBar CS cation-exchange f.p.l.c. column (Flowgen). The IgG was eluted with 50 mM Mes, pH 6.0, containing 200 mM NaCl and further purified by loading on to a 1 ml MonoQ anion-exchange f.p.l.c. column (Pharmacia) in 20 mM triethanolamine and eluting with a linear gradient of 0–350 mM NaCl. The antibody peak was eluted at around 150 mM NaCl. The purity of antibody preparations was confirmed by SDS/PAGE under reducing conditions [14].

Radiolabelling of monoclonal antibodies

MAC188 variants were labelled with ^{125}I (Amersham) and MAC221 was labelled with ^{131}I (Amersham) using lactoperoxidase [15]. Protein concentration was determined by the Lowry method [16].

Animals

Male New Zealand White rabbits (2.5–3.6 kg body weight) or male Dutch rabbits (1.0–2.0 kg body weight) fed a normal chow diet (Biosure R14) *ad libitum* were used.

17 α -Ethynyl oestradiol administration

17 α -Ethynyl oestradiol (Sigma) was dissolved in polyethylene glycol 400 and administered daily to rabbits subcutaneously in a final volume of 0.2 ml/kg body weight in doses ranging from 0.1 to 5.0 mg/kg body weight. Control animals were injected with polyethylene glycol only.

Serum cholesterol measurement

Blood samples were centrifuged at 2300 g for 15 min. Plasma (200 μl) was transferred to sample cups and stored at 4 °C until enzymically assayed for cholesterol concentration using a kit (cholesterol C-system, Boehringer–Mannheim) by means of a COBAS Bio Centrifugal Analyser.

Antibody injection, blood and tissue sampling

Rabbits were sedated by intramuscular injection of 0.2 ml of Hypnorm (fentanyl citrate and fluanisone, Janssen Pharmaceuticals). ^{125}I -labelled MAC188 and ^{131}I -MAC221, both in phosphate-buffered saline, were mixed in equal quantities and injected into the marginal ear vein in a total volume not exceeding 1 ml/kg body weight. The central ear artery was cannulated and blood allowed to run freely for 2 min. Blood samples were taken at various times from the central artery in the contralateral ear and ^{125}I and ^{131}I were measured in an LKB Wallac γ -counter. After 16 min the rabbits were killed by intravenous injection of 400 mg of pentobarbitone sodium (Euthatal, Rhône-Poulenc Rorer). The liver was removed, weighed and samples taken for immunoblotting. Samples of liver, adrenal, kidney, spleen and testis were also taken for γ -counting.

Western blotting of liver extracts

Levels of LDL receptor in liver samples were determined by quantitative Western blotting as described previously [10] with minor modifications. Briefly, liver samples were homogenized and solubilized with buffer containing Triton X100 (15 g/l). Proteins in the detergent extract were then separated on SDS/7.5% polyacrylamide gels [14] under non-reducing conditions and transferred by electroblotting to nitrocellulose. After blocking with BSA (25 g/l) membranes were incubated with ^{125}I -MAC188 (5 $\mu\text{g}/\text{ml}$, 600–1000 c.p.m./ng), then with horseradish peroxidase-conjugated anti-(rat IgG). Bands were visualized with 3',3'-diaminobenzidine tetrahydrochloride substrate and the LDL receptor bands, M_r 120 000 [10], were cut out for γ -counting.

Statistical analysis

Differences between means were compared using Student's *t* test. Statistical significance was inferred when $P < 0.05$.

RESULTS

Variants of MAC188

Isolation of MAC188 variants

The anti-receptor monoclonal MAC188 was produced by fusing Y3 myeloma cells with spleen cells from a rat immunized with purified rabbit receptor [10]. Purified MAC188 was shown by

Figure 1 Separation of MAC188 variants by cation-exchange chromatography

(a) 10 mg of purified MAC188 was loaded on to a 1 ml Mono S column (Pharmacia) in 50 mM Mes, pH 6.0, and was eluted with an NaCl gradient (-----) at 1 ml/min and 0.5 ml fractions were collected. (b) Aliquots from the middle of peaks A, D and F (20 μ g of protein/lane) were run on an SDS/polyacrylamide gel (17.5%) under reducing conditions and stained with Coomassie Blue. The position of heavy chains (H), spleen light chains (S) and Y3 light chains are indicated.

SDS/PAGE (under reducing conditions) to possess two species of light chain. The faster-migrating species was shown to be the Y3 myeloma light chain because it was depleted when the antibody was passed through an immunoaffinity column directed against the Y3 light chain (results not shown). Purified MAC188 was loaded on to a Mono S f.p.l.c. cation-exchange column and eluted with a shallow NaCl gradient. At least six peaks could be detected (Figure 1a). The first peak (A) was shown by SDS/PAGE (Figure 1b) to contain antibody with only the Y3 myeloma light chains (MAC188 Y3/Y3) and the final peak (F) contained antibody with only spleen light chains (MAC188 S/S). The other peaks (Figure 1a) were shown to contain antibody with both spleen and Y3 light chains. It was not possible to determine whether these other peaks contained; (a) pure MAC188 S/Y3, (b) a mixture of MAC188 S/S and MAC188 Y3/Y3, or (c) MAC188 S/Y3 contaminated with one of the other two variants. We were able therefore, to isolate pure MAC188 S/S and MAC188 Y3/Y3 but not able to isolate pure MAC188 S/Y3.

Figure 2 (a) Removal of MAC188 variants and MAC221 from the blood of New Zealand White rabbits and (b) removal of MAC188 S/S and MAC221 from the blood of New Zealand White and Dutch rabbits

(a) Unfractionated MAC188 ($\blacktriangle$), its two variants MAC188 S/S ($\blacksquare$) and MAC188 Y3/Y3 ($\triangle$) and the control antibody MAC221 ($\square$) were radiolabelled and then injected intravenously at 10 μ g/kg body weight. For the first 2 min blood was collected for 5 s every 15 s. Later, 1 ml samples were taken at 5, 10 and 30 min post-injection. Data are expressed as the percentage of injected radioactivity remaining in the blood and represent means (three rabbits for each variant) $\pm$ S.E.M. (b) 125 I-MAC188 S/S was co-injected intravenously with 131 I-MAC221, into rabbits at 10 μ g/kg body weight. For the first 2 min blood was collected for 5 s at 15 s intervals. Later 1 ml samples were taken at 5, 10 and 15 min post-injection to determine the levels of MAC221 (open symbols) and the levels of MAC188 S/S (closed symbols) in New Zealand White ($\square$, $\blacksquare$) and Dutch rabbits ($\circ$, $\bullet$). Data are expressed as the percentage of injected radioactivity remaining in the blood and represent means (four rabbits of each strain) $\pm$ S.E.M.

Disappearance of MAC188 variants following intravenous injection into rabbits

Unfractionated MAC188 and the two pure variants (MAC188 S/S and MAC188 Y3/Y3) were labelled with 125 I, mixed with equal quantities of control antibody (131 I-MAC221) and injected into New Zealand White rabbits at a dose of 10 μ g/kg body weight. Figure 2(a) shows the removal of the antibodies from the blood in the 30 min period after injection. As before [11], removal of the unfractionated antibody was only partial. A fraction of this preparation was removed rapidly (53% within 5 min) but after 30 min 31% still remained in the blood. In contrast, the MAC188 S/S variant was removed much more effectively with 81% cleared in 5 min and 93% within 10 min. There was no receptor-mediated removal of the variant with two myeloma light chains (MAC188 Y3/Y3). This variant behaved in an identical manner to the control antibody MAC221 (Figure 2a), indicating that the heavy chain of MAC188 was not on its own sufficient for high-affinity binding.

MAC188 S/S (10 μ g/kg) was also removed efficiently from the plasma of the smaller Dutch rabbits. There was no difference

Figure 3 Removal of MAC188 S/S and MAC221 from the blood in control and oestrogen-treated rabbit

17α -Ethinyl oestradiol was dissolved in poly(ethylene glycol) and subcutaneously administered daily for 3 days to Dutch rabbits at a dose of 1.0 mg/kg body weight. Control animals were injected with poly(ethylene glycol) only. ^{125}I -MAC188 S/S (filled symbols) was then co-injected with ^{131}I -MAC221 (open symbols). (a) Control rabbits received MAC188 S/S doses of 0.3 mg/kg (Δ , $\blacktriangle$), 1.0 mg/kg ($\square$, $\blacksquare$) and 3.0 mg/kg ($\circ$, $\bullet$). (b) Oestrogen-treated rabbits received MAC188 S/S doses of 1.0 mg/kg ($\square$, $\blacksquare$), 3.0 mg/kg ($\circ$, $\bullet$) and 10.0 mg/kg ($\diamond$, $\blacklozenge$). Blood samples (1 ml) were taken at the times indicated. Data are expressed as the percentage of injected radioactivity remaining in the blood and represent means (six rabbits at each antibody concentration) $\pm$ S.E.M.

between the clearance in New Zealand White and Dutch rabbits (Figure 2b).

Immunoblotting studies

Up-regulation of hepatic LDL receptor by 17α -ethinyl oestradiol

Having purified a fully active preparation of the anti-receptor antibody (MAC188 S/S), the next objective was to use this reagent to measure LDL receptor levels *in vivo* under control conditions and under conditions when known changes in receptor level would occur. 17α -Ethinyl oestradiol was chosen to up-regulate LDL receptor levels [17]. By dosing rabbits with 17α -ethinyl oestradiol and measuring liver LDL receptor protein (using a quantitative Western blotting procedure), we carefully established conditions which would give a defined up-regulation of hepatic LDL receptor protein.

The dose of 17α -ethinyl oestradiol commonly used in the literature (5 mg/kg) was found consistently to reduce body weight in our studies. This side effect, which has been observed before [17], was probably due to reduction in food intake. Short periods of fasting result in increased plasma cholesterol [18] and in an attempt to minimize this, the Western blotting method was

used to study the effect of lower oestrogen doses for a fixed period of 3 days. Up-regulation of receptor protein was observed at doses as low as 0.1 mg/kg and a 2–4-fold increase was noted at 1 mg/kg. Weight reduction was observed in all oestrogen-dosed rabbits, but as this was not dose-dependent a dose of 1 mg/kg as chosen for further studies.

Using MAC188 S/S to measure LDL receptor levels *in vivo*

The next study was designed to measure LDL receptor *in vivo* by co-injecting MAC188 S/S with control antibody MAC221 and then measuring LDL-receptor-mediated removal of antibody from the blood and receptor-mediated uptake by various tissues. When Dutch rabbits (1.5 kg to 2 kg body weight) received 17α -ethinyl oestradiol (1 mg/kg) for 3 days, plasma cholesterol was reduced by 47% ($P < 0.001$). The expected reductions in body weight (9%, $P < 0.001$) and in wet liver weight (24%, $P < 0.001$) were also observed.

Disappearance of MAC188 S/S from the blood

Preliminary studies with untreated animals had shown that, as the injected dose of MAC188 S/S was increased, a dose was reached (less than 2 mg/kg body weight) at which further receptor-mediated clearance ceased, suggesting that all the receptors were occupied. In this study, therefore, doses of MAC188 S/S at 0.3, 1.0 or 3.0 mg/kg were used in control animals. Oestrogen-treated animals were injected with 1.0, 3.0 or 10.0 mg/kg, as the immunoblotting studies (above) had predicted a 2–4-fold increase in hepatic receptor activity following treatment. From the antibody concentrations (30 s after injection), it is possible to estimate that the blood volume accessible to the antibodies was between 58 and 68 ml per kg body weight. This compares well with a blood volume for rabbits of between 57 and 70 ml per kg determined using the Evans Blue dye method [19].

Figure 3 shows the removal of MAC188 S/S and MAC221 in control and treated rabbits over the 15 min period after injection. The removal of MAC221 was not affected by the 17α -ethinyl oestradiol treatment. The results (shown in Figure 3) were then used to calculate the amount of antibody removed by the LDL receptor per kg of body weight (MAC188 S/S removed per kg minus MAC221 removed per kg). The time course of this receptor-mediated removal from the blood is plotted in Figure 4. At all antibody doses, in both control rabbits (Figure 4a) and oestrogen-treated rabbits (Figure 4b), receptor-mediated removal of antibody was essentially complete before the 15 min time point.

Figure 5 illustrates the receptor-mediated removal at the 15 min time point for each dose of antibody. In control animals, the amount of antibody removed by the receptor was not increased when the injected dose was increased from 1.0 mg/kg to 3.0 mg/kg. Full occupancy of the receptors must therefore have occurred at 1.0 mg/kg or lower. In oestrogen-treated rabbits, the amount of antibody removed by the receptor was not increased when the dose was raised from 3.0 mg/kg to 10.0 mg/kg. In this case full occupancy must have occurred at 3.0 mg/kg or lower. A MAC188 S/S dose of 3.0 mg/kg was therefore saturating in both groups. At this dose, the amount of MAC188 S/S removed from the blood by the LDL receptor was 0.51 ± 0.06 mg/kg in controls and 1.0 ± 0.1 mg/kg in oestrogen-treated animals.

Tissue uptake

Figure 6 shows the uptake of MAC188 S/S and MAC221 for kidney, adrenal and liver. It also shows the calculated receptor-

Figure 4 Receptor-mediated removal of MAC188 S/S from the blood, in control and oestrogen-treated rabbits

Receptor-mediated removal of MAC188 S/S from the blood was calculated for the experiments shown in Figures 3(a) and 3(b) by subtracting the amount of control antibody (MAC221) cleared from the amount of MAC188 S/S cleared. (a) Control rabbits received MAC188 S/S doses of 0.3 mg/kg (Δ), 1.0 mg/kg ($\square$) and 3.0 mg/kg ($\bullet$). (b) Oestrogen-treated rabbits received MAC188 S/S doses of 1.0 mg/kg ($\square$), 3.0 mg/kg ($\bullet$) and 10.0 mg/kg ($\diamond$). Receptor-mediated removal was calculated for each rabbit at each time point and for the data illustrated represent means (six rabbits at each antibody concentration).

Figure 5 Total receptor-mediated removal of MAC188 S/S from the blood of control and oestrogen-treated rabbits

The receptor-mediated removal of MAC188 S/S per kg of body weight was calculated from the data shown in Figures 4(a) and 4(b). The levels of control antibody in the blood 30 s after injection were used to determine the amount of blood per kg for each rabbit. The data for control rabbits (○) and oestrogen-treated rabbits (●) are shown and represent means (six rabbits at each antibody concentration) $\pm$ S.E.M.

mediated uptake (MAC188 S/S minus MAC221). The results are expressed in micrograms of antibody per gram of tissue and take no account of the total weight of the respective organs. Receptor-mediated uptake in the kidneys was low (Figures 6a and 6b) and there was no significant increase following oestrogen treatment.

In adrenal (Figures 6c and 6d) and liver tissue (Figures 6e and 6f) receptor-mediated uptake was saturable and, at a saturating

Figure 6 Tissue uptake of MAC188 S/S and MAC221 in control and oestrogen-treated rabbits

Dutch rabbits were treated with control solution or with 17 α -ethynyl oestradiol solution (1.0 mg/kg, for 3 days) and then injected with the indicated doses of 125 I-MAC188 S/S and 131 I-MAC221 (as described in the legend to Figure 3). The rabbits were killed 16 min after injection and samples of tissues removed for γ -counting. The uptake of MAC188 S/S ($\square$), MAC221 (Δ) and the derived receptor-mediated uptake ($\bullet$) are shown in (a) kidney in control animals and (b) kidney in oestrogen-treated animals, (c) adrenals in control animals and (d) adrenals in oestrogen-treated animals, and (e) liver in control animals and (f) liver in oestrogen-treated animals. Data represent means (six rabbits at each antibody concentration) $\pm$ S.E.M.

Figure 7 Total receptor-mediated uptake of MAC188 S/S by the liver of control and oestrogen-treated rabbits

Receptor-mediated uptake of MAC188 S/S per gram of liver (shown in Figures 6e and 6f), was multiplied by liver weight to determine receptor-mediated uptake by whole liver, and then divided by body weight to calculate receptor-mediated uptake of MAC188 S/S per kg body weight. The data for control rabbits (○) and oestrogen-treated rabbits (●) are shown and represent means (six rabbits at each antibody concentration) $\pm$ S.E.M.

Figure 8 Correlation between receptor-mediated removal of MAC188 S/S from the blood and by the liver

The correlation between receptor-mediated removal of MAC188 S/S from the blood and MAC188 S/S uptake by the liver (at the 3 mg/kg dose) is shown for each control (○) and each oestrogen-treated (●) rabbit.

dose of 3.0 mg/kg, oestrogen treatment increased receptor-mediated uptake from $12.4 \pm 2.1 \mu\text{g/g}$ to $20.0 \pm 3.8 \mu\text{g/g}$ in adrenal tissue ($P < 0.1$) and from $11.0 \pm 1.0 \mu\text{g/g}$ to $23.0 \pm 2.1 \mu\text{g/g}$ in liver ($P < 0.01$). The mean liver weight in control animals was $69.2 \pm 3.5 \text{ g}$, and it was $52.8 \pm 2.3 \text{ g}$ in treated animals. The mean adrenal weight was $61.7 \pm 13.8 \text{ mg}$ in control animals and $66.3 \pm 18.6 \text{ mg}$ in treated animals.

There was no significant receptor-mediated uptake in the testis or spleen in either control or oestrogen-treated animals (results not shown).

In this study, 36 rabbits (body weight 1.5 kg to 2.0 kg) were used and liver weights varied from 37 g to 95 g. Figure 7 shows the receptor-mediated uptake of MAC188 S/S by the whole liver (corrected for body weight). The data indicate that uptake by the liver was responsible for approximately 80% of the removal from the blood. Following the saturating dose of 3.0 mg/kg body weight, receptor-mediated uptake of MAC188 S/S by the liver was $0.41 \pm 0.08 \text{ mg/kg body weight}$ in control animals, and $0.78 \pm 0.08 \text{ mg/kg}$ in treated animals.

When a saturating dose of MAC188 S/S was administered there was a good correlation between receptor-mediated removal from the blood and receptor-mediated uptake by the liver. Figure 8 shows the data for both control and oestrogen-treated rabbits at the 3.0 mg/kg dose. The value for r (the correlation coefficient) was 0.82.

DISCUSSION

In a previous study we showed that the monoclonal antibody MAC188 could be used as a probe for the LDL receptor *in vivo* [11]. The antibody, as secreted from myeloma cells, however, is comprised of three variants of which one has two inactive light chains and does not bind to the LDL receptor. This complicates blood clearance studies as the inactive variant remains in the plasma contributing to high experimental background. In the present study we have separated the three variants of MAC188 and shown that the species with two active light chains (MAC188 S/S) is completely and rapidly removed from the blood.

The binding and trapping properties of this MAC188 S/S variant make it an ideal reagent to measure the absolute number of functioning LDL receptors or 'LDL receptor status' of a rabbit. Following injection, the amount of MAC188 S/S specifically removed from the blood represents the amount of antibody sequestered by receptors inside cells plus the amount bound to receptors on the cell surface. If the administered dose of MAC188 S/S is high enough to occupy all the available receptors in an animal (saturation) then the amount of antibody removed from the blood can be related to the total number of accessible receptors in that animal. In order to demonstrate the suitability of MAC188 S/S for this purpose, we used the antibody to measure LDL receptor levels under control conditions and following treatment with 17α -ethinyl oestradiol.

17α -Ethinyl oestradiol up-regulates LDL receptor activity *in vivo* by increasing the level of receptor mRNA [17]. Using a quantitative Western blotting technique on rabbit liver extracts, we first determined which dose of 17α -ethinyl oestradiol would approximately double hepatic receptor expression. Rabbits were treated with this dose of oestrogen (1 mg/kg for 3 days) after which they were injected with various amounts of a mixture of MAC188 S/S and the control antibody MAC221. The amount of MAC188 S/S removed from the blood by the receptor was then calculated for each dose. Saturation of the receptor-mediated removal occurred in both control and treated groups after 3.0 mg/kg of MAC188 S/S was injected. At this dose, the amount of antibody cleared specifically was 0.5 mg ($3.3 \times 10^{-9} \text{ mol}$)/kg in control animals and 1.0 mg ($6.6 \times 10^{-9} \text{ mol}$)/kg in treated animals.

Binding studies on rabbit fibroblasts using MAC188 (the unfractionated antibody) indicated that the stoichiometry of antibody binding to the receptor is 1:1 [11]. Based on these data and on the blood clearance results at 3.0 mg/kg, the total number of accessible LDL receptors (or LDL receptor status) can be

calculated as $(2.0 \pm 0.3) \times 10^{15}$ receptors per kg in control rabbits and $(4.0 \pm 0.4) \times 10^{15}$ receptors per kg in oestrogen-treated animals.

At saturating doses of MAC188 S/S, receptor-mediated uptake of the antibody by the liver was shown to be 0.41 mg $(2.7 \times 10^{-9}$ mol)/kg in control animals. Thus receptor-mediated uptake in the liver accounted for 80 % of the receptor-mediated removal from the blood. This is in good agreement with Spady et al. [3], who found that normal rabbit liver contained 83 % of total receptor-dependent LDL transport. Following oestrogen treatment in this study, receptor-mediated uptake by the liver was increased from 0.41 to 0.78 mg/kg body weight. From these data, the mean receptor number in the liver can be calculated as $(1.6 \pm 0.3) \times 10^{15}$ receptors per kg body weight in control rabbits and $(3.1 \pm 0.3) \times 10^{15}$ receptors per kg in treated rabbits.

The maximal rate at which LDL can be transported out of the plasma by LDL receptors (denoted J^m by Dietschy et al. [1]) is proportional to the total number of LDL receptors [1]. Although this related parameter has already been determined for several tissues [2,3,20], it has not previously been possible to measure the absolute numbers of functioning receptors in a given organ. This is now possible using MAC188 S/S.

Having determined the total number of LDL receptors in the liver it should be possible, by making certain assumptions, to calculate the number of receptors in each hepatocyte. The number of hepatocytes in rat liver has been estimated as 1.3×10^8 /g [21]. Assuming that rabbits and rats have a similar number, it can be calculated that the mean hepatocyte number for control rabbits in the current study was approximately 4.6×10^9 cells/kg body weight. Using our figure of 1.6×10^{15} LDL receptors/kg body weight and assuming that only parenchymal cells have LDL receptors, it can be estimated that the maximum number of receptors per hepatocyte in control animals would be 3.6×10^5 . It is difficult to compare this figure with those obtained using hepatocytes in culture. The number of receptors on cultured cells varies with species, cell type and conditions. Thus, the number of cell-surface receptors for LDL may be virtually zero when normal cells are incubated with sterols [22] but has been reported to be as high as 7×10^4 per cell on human fibroblasts [23] and 6×10^4 per cell on Hep G2 cells [24] in the absence of sterols. Since only 25 % of LDL receptors appear to be on the cell surface at any one time [25], the total number of functioning receptors in cells in culture may range between 0 and 2.8×10^5 per cell.

In vivo, the rate of receptor-mediated transport of LDL cholesterol out of the plasma is related to the number of LDL receptors. This rate (J^m) has been determined in rabbits as 2.13 mg of cholesterol/h per kg [26]. Assuming that the liver is responsible for 80 % of LDL receptor activity, J^m for the rabbit liver can be calculated as 1.7 mg of cholesterol/h per kg. Making the assumption that the cholesterol moiety represents 30 % (by weight) of the LDL particle and that ApoB (M_r 500000) represents 21 %, it can be calculated that the LDL receptors in rabbit liver must transport 1.5×10^{15} LDL particles/h per kg body weight. This is very close to the number of receptors (1.6×10^{15} /kg body weight) determined using MAC188 S/S in our study. To calculate the absolute number of receptors from the rate of LDL transport it would be necessary to know the rate of receptor recycling for the rabbit *in vivo*. In human fibroblasts in culture, receptors are believed to recycle every 12 min [25], but the recycling time for receptors in rabbit hepatocytes is not known.

Spady et al. [3] reported that the rate of receptor-dependent transport of LDL in rabbit adrenal tissue was ten times higher than that in liver. In the current study receptor-mediated uptake

of MAC188 S/S per gram of tissue was approximately the same for the liver and adrenals. This means that the number of receptors per gram of tissue was the same for both organs. If this is the case, the higher rate of receptor-dependent LDL transport reported for adrenal tissue [3] is not simply due to a higher density of LDL receptors in that tissue.

Recently, a rabbit VLDL receptor cDNA has been sequenced and this protein shows a remarkable degree of sequence similarity with the rabbit LDL receptor [27]. Given the similarities between the two, it could not be ruled out that MAC188 could bind to both receptors. The pattern of tissue uptake for MAC188, however, indicated that this is probably not the case. The mRNA for the putative VLDL receptor is most abundant in the heart [27] where MAC188 binding is negligible [11] and the VLDL receptor is virtually absent from the liver where MAC188 binding is highest.

Another membrane protein which could potentially bind to MAC188 S/S is the LDL receptor-related protein (LRP). This molecule has domains homologous to those of the LDL receptor, and like the LDL receptor, most LRP activity appears to be in the liver [28]. Although we cannot discount some interaction between MAC188 S/S and LRP, we have never observed the 515000 M_r subunit of LRP on Western blots of liver extracts probed with MAC188.

It is of obvious importance to be able to measure changes in LDL status induced by drugs, diet and disease. These are circumstances where complex changes in the concentration and composition of competing lipoproteins make it difficult to use LDL itself as a probe for the LDL receptor. The antibody MAC188 S/S does not have these disadvantages and represents an ideal reagent for studying changes in total receptor number in the rabbit. If an antibody to the human LDL receptor, with properties similar to MAC188, can be developed it could prove useful in clinical studies on hypercholesterolaemia.

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