Probing of the expression of the low-density lipoprotein receptor *in vivo* using an anti-receptor monoclonal antibody

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MAC188 is a rat anti-[low-density lipoprotein (LDL) receptor] monoclonal antibody (McAb) which binds to the cell surface receptor with high affinity at physiological temperatures, even in the presence of high concentrations of the natural ligand, LDL. Binding of McAb MAC188 at 37 °C is followed by internalization and intracellular sequestering of the receptor, which results in the transient disappearance of the receptor from the cell surface. The high binding affinity and epitope specificity of McAb MAC188 suggested that this antibody could be used to quantify receptor expression *in vivo*. Mixtures of radiolabelled anti-receptor antibody and a control McAb (MAC221) were injected intravenously into rabbits, and the clearance from serum and uptake into tissues was determined. A fraction of the anti-receptor McAb was cleared rapidly from the circulation by a high-affinity and saturable (receptor-dependent) process. Receptor-dependent uptake of the anti-receptor McAb was measurable in liver, adrenal glands, kidneys, spleen, kidney, thoracic aorta and heart. It was highest in liver and adrenal glands and correlated well with the level of receptor protein and the rate of LDL transport in individual tissues. Anti-receptor McAbs such as MAC188, with suitable domain specificity and binding affinity at physiological temperatures, have important advantages over the natural ligand as tracers for the receptor *in vivo*, and may find widespread applications in studies of the receptor status (activity) in animals and man.

INTRODUCTION

The ability to quantify the expression of the low-density lipoprotein (LDL) receptor *in vivo* is of considerable value. It is now firmly established that most LDL receptors are located in the liver and that changes in hepatic LDL receptor activity produce changes in plasma LDL levels in animals (Kovanen *et al.*, 1981; Spady & Dietschy, 1985, 1988) and man (Starzl *et al.*, 1984). However, the quantification of liver LDL receptors in man remains problematic and has relied so far on receptor assays using liver biopsies (Soutar *et al.*, 1986). In other studies, receptor levels in blood mononuclear cells have been quantified in an attempt to establish a correlation with whole-body (largely hepatic) LDL receptor levels (Turner *et al.*, 1984), but it is not yet clear to what extent the expression of the LDL receptor in extra-hepatic cells reflects LDL receptor activity in liver.

Based on the observation that certain chemical modifications of LDL abolish receptor-dependent (but not receptor-independent) LDL clearance, the contributions of both the receptor-dependent and receptor-independent pathways of LDL clearance *in vivo* have been measured in experimental animals using mixtures of radiolabelled native and chemically modified LDL (Pittman *et al.*, 1979, 1982; Slater *et al.*, 1980; Carew *et al.*, 1982; Spady *et al.*, 1983, 1985, 1987). Similar studies in man (Kesaniemi *et al.*, 1983) have provided estimates of receptor-mediated LDL clearance in substantial agreement with the values predicted from LDL turnover studies in subjects with homozygous familial hypercholesterolaemia (FH) (Bilheimer *et al.*, 1979). These patients lack functional LDL receptors and clear LDL from plasma solely by the non-receptor pathway.

In a more recent approach, Dietschy and co-workers have sought to derive the rate of receptor-dependent clearance of LDL in man from simple turnover studies of native LDL. Using data from studies in animals and FH patients, and assuming that the binding affinity of LDL for the receptor does not differ in animals and man, Meddings & Dietschy (1986) derived equations which allow the calculation of whole-body LDL receptor activity from LDL turnover studies. These authors also concluded from their study that the total number of LDL receptors is the most important determinant of the rate of LDL clearance in man.

Despite the obvious relevance of using LDL as a ligand for probing the LDL receptor *in vivo*, there are potential limitations. A major limitation resides in the heterogeneity of the ligand. There is ample evidence that the conventionally defined LDL fraction includes a spectrum of lipoprotein particles which vary considerably in size and chemical composition and, more significantly, in their relative ability to bind the LDL receptor (Knight *et al.*, 1986a). This limitation is even more severe in situations where high concentrations of abnormal lipoproteins accumulate in the plasma of animals (Witztum *et al.*, 1985) or patients with hyper- or dys-lipoproteinaemia. Yet these are precisely the cases where it would be important to establish whether changes in receptor levels play a role in the origin of the disorder.

Anti-(LDL receptor) monoclonal antibodies (McAbs) of suitable affinity and domain specificity may represent more appropriate probes for the LDL receptor *in vivo*, but few McAbs have so far met the necessary requirements (Huettinger *et al.*, 1984). Here we report the further characterization and the use of

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; McAb, monoclonal antibody; FH, familial hypercholesterolaemia; LDFCS, lipoprotein-deficient fetal calf serum; LDL, low-density lipoprotein ($\rho = 1.019\text{--}1.063\text{ g/ml}$); TBS, Tris-buffered saline (0.025 M-Tris/HCl/0.15 M-NaCl, pH 7.4); PBS, phosphate-buffered saline (0.025 M-NaH₂PO₄/Na₂HPO₄/0.15 M-NaCl, pH 7.4).

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the anti-(LDL receptor) McAb MAC188 (Gherardi *et al.*, 1988b) as a tracer for the LDL receptor *in vivo*.

MATERIALS AND METHODS

Animals

Male New Zealand White rabbits (1.6–2.7 kg) were used. Radiolabelled McAbs were injected into the ear marginal vein. McAbs were adjusted to 10 µg/ml in phosphate-buffered saline (PBS) containing 5 mg of BSA/ml. Blood samples were collected at various times from the contralateral ear vein. Blood was transferred to 1.5 ml Eppendorf tubes, allowed to clot, centrifuged (800 g, 15 min) and ^{125}I and ^{131}I radioactivity was measured in an LKB Wallac γ -radiation counter. In order to assess the amount of antibody taken up in various tissues, the following procedure was used. Animals were anaesthetized with a subcutaneous injection of Hypnorm (Janssen Pharmaceuticals; 0.25 ml/kg) followed by an injection of Althesin (Glaxo Laboratories Ltd.; 50–100 µg/kg). The left external carotid artery was exposed and cannulated, and the animal was perfused with a solution of PBS containing BSA (6 mg/ml) at room temperature at a pressure of 80–90 mmHg (10.7–12.0 kDa). At the moment that perfusion commenced, the animal was killed by an intravenous injection of Euthatal (May & Baker; 0.5 ml/kg). A femoral artery was then cannulated and the perfusate was allowed to flow from this cannula for 15 min. After this time the perfusion was stopped, the viscera were exposed through a ventral mid-line incision and the various tissues were sampled.

Serum LDL

LDL was prepared from rabbit and human serum as described (Gherardi *et al.*, 1988a). Lipoproteins were stored in 0.01 M-Tris/HCl/0.001 M-EDTA, pH 7.4, at 4 °C and used within 3 weeks of isolation and 1 week of iodination. Iodination was carried out using a modification of the lactoperoxidase procedure of Marchalonis (1969).

Purification and labelling of McAbs

Two McAbs were used in this study. The anti-(LDL receptor) McAb MAC188 (Gherardi *et al.*, 1988b) is a rat IgG1 obtained by fusion of the spleen cells from a DA rat immunized with purified rabbit LDL receptor and the L-chain-secreting rat myeloma line Y3 Ag1.2.3 (Galfré *et al.*, 1979). The control McAb MAC221 is a rat IgG1 obtained by fusion of the spleen cells from a F344 rat immunized with bovine placental lactogen and the IR983F myeloma (Forsyth *et al.*, 1987). Internal labelling experiments with [^{14}C]lysine showed that both the anti-receptor and control McAbs are secreted as molecular mixtures containing either the spleen and/or the myeloma light chains. The hybridoma line secreting McAb C7 (Beisiegel *et al.*, 1981) was obtained from the American Type Culture Collection. McAbs were purified from ascitic fluid as described previously (Gherardi *et al.*, 1988a) and their purity was checked on SDS/15%-polyacrylamide gels under both reducing and non-reducing conditions. McAbs were labelled at specific radioactivities of 500–1000 c.p.m./ng with ^{125}I or ^{131}I using the same modification of the lactoperoxidase procedure used for the iodination of LDL.

Binding of McAbs to rabbit fibroblast monolayers

Binding of ^{125}I -McAb to rabbit fibroblasts was carried out as described (Gherardi *et al.*, 1988a). Briefly, 25000 cells were plated in 35 mm-diam. dishes and cultured for 4 days in 10% fetal calf serum in Dulbecco's modified Eagle's medium (DMEM) and for 1 day in lipoprotein-deficient fetal calf serum (LDFCS). On day 5, cells were washed twice with PBS and incubated with

^{125}I -McAb or ^{125}I -LDL at either 4 °C or 37 °C as detailed in the legend to Figs. 1–5. The amount of ^{125}I -McAb or ^{125}I -LDL degraded by cells in culture or *in vivo* was measured as trichloroacetic-acid-soluble radioactivity, as described by Goldstein & Brown (1974). Apparent K_d and B_{max} values of the high-affinity binding of McAb MAC188 and LDL to the fibroblast receptor were derived by Scatchard analysis (Scatchard, 1949).

Other assays

LDL receptor blots on rabbit tissues were carried out as described (Gherardi *et al.*, 1988b). Protein was assayed using a modification of the Lowry procedure (Markwell *et al.*, 1978).

RESULTS

Binding of McAb MAC188 to the fibroblast LDL receptor at 4 °C and 37 °C

Initial experiments showed that McAb MAC188 bound with high affinity to purified rabbit LDL receptors (half-maximal binding at ~4 pmol/ml; maximal binding at ~16 pmol/ml) and that binding of the anti-receptor McAb (unlike binding of the natural ligand, LDL) did not require the presence of divalent cations (Gherardi, 1987). These results suggested that the epitope recognized by McAb MAC188 may not be contained in the LDL-binding site and led us to investigate further the binding of ^{125}I -MAC188 and ^{125}I -LDL to the native receptor. Figs. 1 and 2 show the total (closed symbols) and low-affinity (open symbols) binding of ^{125}I -MAC188 and ^{125}I -LDL to rabbit fibroblast monolayers at 4 °C (Fig. 1) and 37 °C (Fig. 2) after a 2 h incubation period. Several important differences were apparent between the two ligands. (i) At both temperatures the low-affinity (non-specific) binding of the anti-receptor McAb was lower. (ii) Whereas the apparent binding affinity of the natural ligand was significantly lower at 37 °C than at 4 °C, that of ^{125}I -MAC188 did not change appreciably (K_d = 5.5 and 3.3 pmol/ml at 4 °C and 37 °C respectively). Thus at 37 °C the binding affinity of ^{125}I -

Fig. 1. Isotherms of binding of ^{125}I -MAC188 and ^{125}I -LDL to rabbit fibroblast monolayers at 4 °C

Cells (25000/35 mm-diam. dish) were cultured for 4 days in 10% FCS and for 1 day in 10% LDFCS. At day 5, cultures were washed with PBS and incubated in 10% LDFCS in DMEM/Hepes for 1 h before addition of (a) ^{125}I -MAC188 (196 c.p.m./ng) or (b) ^{125}I -LDL (70 c.p.m./ng) at the final concentrations indicated for measuring total binding (●, ■). Low-affinity binding (○, □) was measured in duplicate dishes containing the indicated concentration of radioactive ligands plus 1 µM unlabelled ligand. Cells were incubated with the radioactive ligands for 2 h at 4 °C, with constant rocking, and then washed for 3 × 5 min with 2.5 mg of BSA/ml in PBS, followed by a 5 min wash with PBS. Radioactivity bound to the cells was measured after solubilization with SDS (10 mg/ml). Each point represents the mean value of duplicate dishes.

Fig. 2. Isotherms of binding of ^{125}I -MAC188 and ^{125}I -LDL to rabbit fibroblast monolayers at 37 °C

The same probes used in the experiment reported in Fig. 1 were used, and experimental conditions were the same except that cells were incubated for 2 h at 37 °C with (a) ^{125}I -MAC188 or (b) ^{125}I -LDL at the final concentrations indicated, with constant rocking. After the 2 h incubation, cells were transferred on ice and washed with ice-cold buffers as indicated in the legend to Fig. 1 before measuring cell-associated radioactivity. Each point represents the mean value of duplicate dishes. ●, ▲, Total binding; ○, △, non-specific binding.

Table 1. Inhibition of LDL receptor recycling by McAb MAC188

Cultures of rabbit fibroblasts were set up as described in the legend to Fig. 1. Cells were incubated at 37 °C for 0.5–4 h with McAb MAC188 (32 pmol/ml) or for 1 h with NH_4Cl (10 mM) before cooling to 4 °C. Binding of ^{125}I -LDL (400 c.p.m./ng) at 4 °C was carried out as described in the legend to Fig. 1. Receptor-dependent binding was calculated by subtracting the binding of ^{125}I -LDL in the presence of 1 μM unlabelled LDL from the total binding. Values shown are the means $\pm$ S.D. of triplicate dishes.

Treatment	^{125}I -LDL binding (fmol/mg)	
	Receptor-dependent	Receptor-independent
None	68.8 $\pm$ 1.9	22.6 $\pm$ 0.9
MAC188 (30 min)	28.0 $\pm$ 3.1	20.4 $\pm$ 1.2
MAC188 (1 h)	21.4 $\pm$ 1.2	22.8 $\pm$ 0.3
MAC188 (2 h)	14.4 $\pm$ 6.2	24.2 $\pm$ 4.7
MAC188 (3 h)	4.6 $\pm$ 7.8	26.0 $\pm$ 5.9
MAC188 (4 h)	2.8 $\pm$ 3.8	24.1 $\pm$ 1.6
NH_4Cl (1 h)	32.4 $\pm$ 4.8	23.4 $\pm$ 2.2

MAC188 for the receptor was 15–20-fold higher than that of LDL ($K_d = 56$ pmol/ml). (iii) The decrease in the apparent binding affinity of the natural ligand at 4 °C compared with that at 37 °C was paralleled by a 8–10-fold increase in the B_{max} . However, the B_{max} for ^{125}I -MAC188 binding at 37 °C was approximately twice the value at 4 °C (309 versus 144 pmol/mg). These results suggested that McAb MAC188 was internalized (as the amount of cell-associated ^{125}I -MAC188 was higher at 37 °C than at 4 °C), but that the rate of internalization and/or degradation of the anti-receptor McAb differed from that of LDL.

To address this point, the time courses of binding uptake and degradation of ^{125}I -MAC188 and ^{125}I -LDL were compared at a

Fig. 3. Time course of binding/internalization (a, c) and degradation (b, d) of ^{125}I -MAC188 (a, b) and ^{125}I -LDL (c, d) by rabbit fibroblast monolayers at 37 °C

Cultures were prepared as described in the legend to Figs. 1 and 2 and incubated with 32 pmol of ^{125}I -MAC188 (295 c.p.m./ng) or ^{125}I -LDL (100 c.p.m./ng)/ml for the length of time indicated in the Figure, with constant rocking. After incubation, a 0.5 ml sample of culture supernatant was placed on ice for measuring the amount of ^{125}I -MAC188 or ^{125}I -LDL degraded (Goldstein & Brown, 1974). Cells were washed and cell-associated radioactivity was counted as described in the legend to Fig. 1. Each point represents the mean value of duplicate dishes. ●, ▲, Total; ○, △, non-specific.

fixed concentration of the two ligands. Cells were incubated with 32 pmol of ^{125}I -MAC188 or ^{125}I -LDL/ml (a concentration of ^{125}I -MAC188 which saturated receptor binding; see Fig. 2) and the amounts of ^{125}I -ligands bound (Figs. 3a and 3c) or degraded (Figs. 3b and 3d) were quantified. The experiment indicated that the rates of both internalization and degradation of the anti-receptor McAb were lower than those for LDL, and that degradation of the anti-receptor McAb was negligible for the first 3 h of incubation.

Inhibition of LDL receptor recycling by McAb MAC188

We hypothesized that the low rate of internalization and the lack of degradation of McAb MAC188 over the first 3 h of incubation could be associated with trapping of the receptor inside the cells by the McAb. Several agents, such as monensin (Basu *et al.*, 1981) and NH_4Cl (Grant *et al.*, 1990) are known to inhibit the transport and recycling of the LDL receptor.

In order to investigate receptor recycling, rabbit fibroblasts were incubated at 37 °C with McAb MAC188 at 32 pmol/ml for 0.5–4 h. Unbound antibody was then removed by washing, the cells were cooled to 4 °C and cell surface LDL receptors were probed with ^{125}I -LDL. The results from a typical experiment are shown in Table 1. Incubation of the cells for 30 min with McAb

Fig. 4. Competition by LDL for binding of McAbs ^{125}I -MAC188 and ^{125}I -C7 to rabbit and human fibroblast LDL receptors

The experiments were set up as detailed in the legends to Figs. 1 and 2. Rabbit cells were incubated for 2 h with 2 pmol of ^{125}I -MAC188/ml (343 c.p.m./ng) at 4 °C (○) or with 4 pmol of ^{125}I -MAC188/ml (196 c.p.m./ng) at 37 °C (●) plus rabbit LDL (0–4096 pmol/ml). Human cells were incubated for 2 h at 37 °C with 4 pmol of ^{125}I -C7/ml (390 c.p.m./ng) plus LDL (0–4096 pmol/ml). For comparison, the data are expressed as percentages of binding of the two McAbs in the absence of LDL. This was 75.8 fmol/mg (^{125}I -MAC188 at 4 °C), 316.0 fmol/mg (^{125}I -MAC188 at 37 °C) and 549.8 fmol/mg (^{125}I -C7 at 37 °C). Low-affinity binding was 10.6, 11.6 and 41.2 fmol/mg respectively. Each point represents the mean value of duplicate dishes.

Fig. 5. Clearance of ^{125}I -MAC188 and ^{131}I -MAC221 from rabbit serum

^{125}I -MAC188 (●, 364 c.p.m./ng) and ^{131}I -MAC221 (■, 274 c.p.m./ng) were diluted in 5 mg of BSA/ml in PBS to 10 $\mu\text{g}/\text{ml}$ and injected into the ear marginal vein at a dose of 10 $\mu\text{g}/\text{kg}$. Blood samples were collected from the contralateral ear at the times shown and data are expressed as the percentage of the injected amount of radioactivity remaining in the serum. Results are means $\pm$ S.D. from three rabbits.

MAC188 was sufficient to decrease the number of surface receptors by $\sim 60\%$, and after 3–4 h $> 90\text{--}95\%$ of the receptors had disappeared from the cell surface. In the same experiment

NH_4Cl decreased the number of cell surface receptors by $\sim 50\%$. In other experiments it was found that the number of surface receptors had returned to normal by 4 h after removal of the antibody from the medium (results not shown).

Binding of McAb MAC188 to the LDL receptor in the presence of LDL

Fig. 4(a) shows the binding of ^{125}I -MAC188 to rabbit fibroblast LDL receptors in the presence of LDL. Experiments were carried out at 4 °C and 37 °C at a concentration of McAb of 2 nM (4 °C) or 4 nM (37 °C), and the LDL concentrations were such as to produce the LDL/McAb molar ratios indicated in the Figure. LDL decreased the binding of ^{125}I -MAC188 by $\sim 15\%$ at 4 °C and by 25–30% at 37 °C, and in both cases inhibition was maximal at a molar ratio of LDL/McAb of 256 (Fig. 4a). This corresponded to a concentration of LDL in the medium which saturated receptor binding both at 4 and 37 °C (Figs. 1 and 2). No further inhibition was observed at higher concentrations of the natural ligand. In other experiments we found that binding of ^{125}I -LDL to rabbit fibroblasts was not inhibited by up to a 1024-fold molar excess of McAb MAC188 (results not shown). We therefore conclude that McAb MAC188 and LDL bind the receptor independently and simultaneously. The inhibition of binding of ^{125}I -MAC188 in the presence of LDL is very limited compared with what is observed with other anti-receptor McAbs. For example, McAb C7 [an anti-(bovine LDL receptor) McAb which cross-reacts with the human receptor] is known to bind the receptor with high affinity (and without competition by LDL) at 4 °C, but with much lower affinity at 37 °C (Beisiegel *et al.*, 1981). Interestingly, competition by LDL was much more pronounced at 37 °C (Fig. 4b), a result which highlights the role of antibody affinity in receptor binding at a physiological temperature.

Clearance and tissue uptake of anti-receptor McAb MAC188 *in vivo* in the rabbit

The binding features of McAb MAC188 at physiological temperatures (high affinity and limited competition by the natural ligand) encouraged us to test this antibody *in vivo*. ^{125}I -MAC188 was injected into rabbits together with a control IgG1 (^{131}I -MAC221) directed against an irrelevant antigen and which showed no detectable binding to the fibroblast LDL receptor (results not shown). Fig. 5 shows typical results of the serum clearance of the two antibodies injected at a dose of 10 $\mu\text{g}/\text{kg}$ body weight. The concentration of ^{125}I -MAC188 in serum showed a rapid decline during the first 10–15 min, after which the clearance curve of the anti-receptor antibody followed closely that of the control antibody. Co-injection of unlabelled MAC188 (500 $\mu\text{g}/\text{kg}$) with ^{125}I -MAC188 (10 $\mu\text{g}/\text{kg}$) abolished completely the rapid clearance of ^{125}I -MAC188 (Fig. 6a). This confirmed the receptor-mediated nature of the rapid clearance of ^{125}I -MAC188 from serum and indicated that a dose of 500 μg of MAC188/kg was sufficient to saturate the body pool of LDL receptors in the rabbit. Co-injection of unlabelled MAC221 (500 $\mu\text{g}/\text{kg}$) with ^{125}I -MAC221 (10 $\mu\text{g}/\text{kg}$), as expected, had no effect on the clearance curve of the control antibody (Fig. 6b).

The tissue binding and uptake of the anti-receptor and control McAbs 1 h after injection of the tracers is shown in Table 2. Although the highest ratio of total/non-receptor-dependent uptake of ^{125}I -MAC188 was found in the adrenal glands, the liver showed the highest absolute uptake of the anti-receptor McAb. The other tissues studied showed lower, but measurable, receptor-dependent binding/uptake of ^{125}I -MAC188. However, in the abdominal aorta the specific uptake of ^{125}I -MAC188 was very low, and in the lung it was not measurable in two animals out of three, and was also very low in the third animal.

Fig. 6. Clearance of ^{125}I -MAC188 and ^{125}I -MAC221 from rabbit serum: effect of unlabelled antibody

(a) ^{125}I -MAC188 (147 c.p.m./ng) and (b) ^{125}I -MAC221 (151 c.p.m./ng) were injected separately at $10\text{ }\mu\text{g/kg}$ into four rabbits. The first rabbit (●) received $10\text{ }\mu\text{g}$ of ^{125}I -MAC188/kg, the second (○) received $10\text{ }\mu\text{g}$ of ^{125}I -MAC188/kg + $500\text{ }\mu\text{g}$ of unlabelled MAC188/kg, the third (■) received $10\text{ }\mu\text{g}$ of ^{125}I -MAC221/kg, and the fourth (□) received $10\text{ }\mu\text{g}$ of ^{125}I -MAC221/kg + $500\text{ }\mu\text{g}$ of unlabelled MAC221/kg. Data are expressed as percentages of the injected radioactivity remaining in the serum.

Fig. 7. Receptor-dependent uptake of McAb ^{125}I -MAC188 and $[^{14}\text{C}]$ sucrose-LDL in rabbit tissues

Data are from Table 2 in this work and from Table 2 in Spady *et al.* (1987). Receptor-dependent uptake of ^{125}I -MAC188 was calculated as the difference between the uptake of ^{125}I -MAC188 and that of ^{131}I -MAC221. Receptor-dependent uptake of rabbit $[^{14}\text{C}]$ sucrose-LDL was calculated as the difference between the uptake of $[^{14}\text{C}]$ sucrose-LDL and that of $[^{14}\text{C}]$ sucrose-methyl-human LDL. Tissues used: ●, heart; ■, kidneys; □, spleen; △, liver; ○, adrenal gland. $r = 0.565$.

Receptor-dependent uptake of ^{125}I -MAC188 correlated well with previous estimates (Gherardi, 1987; Gherardi *et al.*, 1988b) of the amount of LDL receptor protein in rabbit tissues (results not shown). It also correlated with the rates of receptor-dependent transport of $[^{14}\text{C}]$ sucrose-LDL in rabbit tissues measured by Dietschy and colleagues (Spady *et al.*, 1987) (Fig.

Table 2. Binding/uptake of ^{125}I -MAC188 and ^{131}I -MAC221 in rabbit tissues

Data were obtained from the same animals used in the experiment shown in Fig. 5. At 1 h after injection of the mixture of ^{125}I -MAC188 and ^{131}I -MAC221, tissues were perfused and tissue-associated radioactivity was measured (see the Materials and methods section for details). Receptor-dependent uptake of ^{125}I -MAC188 was calculated as the difference between the uptake of ^{125}I -MAC188 and that of ^{131}I -MAC221. Values are the means $\pm$ S.D. of three animals.

Tissue	Binding/uptake (ng/g)		Receptor-dependent
	^{125}I -MAC188	^{131}I -MAC221	
Small intestine	2.4 ± 1.6	3.0 ± 2.2	0
Spleen	7.4 ± 2.8	3.8 ± 2.3	3.6
Adrenals	30.7 ± 6.2	3.3 ± 2.1	27.4
Kidneys	6.5 ± 1.1	2.3 ± 0.4	4.2
Liver	54.8 ± 10.9	17.0 ± 4.5	37.8
Lungs	5.7 ± 1.9	5.9 ± 2.4	0
Heart	2.6 ± 0.5	1.9 ± 0.3	0.7
Thoracic aorta	2.1 ± 0.4	0.8 ± 0.7	1.3
Abdominal aorta	2.1 ± 0.5	2.4 ± 1.1	0

7). Several differences, nevertheless, were clear between the results obtained with the anti-receptor McAb and LDL in certain tissues. For example, the highest rate of uptake of ^{125}I -MAC188 was observed in liver, whereas the highest rate of transport of $[^{14}\text{C}]$ sucrose-LDL was found in the adrenal glands (Fig. 7). Furthermore, receptor-dependent uptake of ^{125}I -MAC188 was negligible in the lungs (Table 2), whereas that of $[^{14}\text{C}]$ sucrose-LDL was clearly measurable (Spady *et al.*, 1987). There are several explanations for the observed differences, including the possibility of unequal probe distribution (the molecular masses of the two tracers are very different), the different experimental protocols (bolus injection of the anti-receptor antibody versus constant infusion of LDL) and, probably most important, the effect of McAb MAC188 on receptor recycling (see above). It is clear, nevertheless, that the differences between the tissue uptake of the anti-receptor McAb and LDL will require further studies.

DISCUSSION

In the case of receptor systems in which the ligand is a single, chemically homogeneous species, the ligand itself may represent a suitable tracer for the quantification of receptor numbers *in vivo*. There are, of course, important additional prerequisites to be met (such as knowledge of the concentration of the endogenous ligand, of putative inhibitors or carriers, etc.), but in principle the natural ligand may, in these systems, act as a quantitative tracer for the receptor.

Lipoprotein receptors (one of which, the LDL receptor, is of crucial importance in the regulation of cholesterol supply in the body, and especially in the liver) fall, however, into a different category, in that there are multiple ligands for each receptor, and both the concentrations and binding affinities of the different ligands vary between individuals (and in each individual) depending on the genetic, dietary and hormonal background. It is our opinion that anti-receptor McAbs may be superior to the natural ligand(s) as tracers for such receptors *in vivo*, and we have shown in this work that a McAb of suitable affinity and domain specificity can be derived for this application.

McAb MAC188 binds the LDL receptor at physiological temperature with very high affinity (one to two orders of

magnitude higher than that of LDL) and in the presence of high concentrations of the natural ligand such as those found *in vivo*. Once internalized, this McAb remains associated with the receptor for at least several hours before being degraded, and when all receptors have associated with the McAb on the cell surface and moved inside the cells, the number of receptors found on the cell surface is markedly decreased (Table 1). This behaviour was unexpected and, to our knowledge, so far unreported for other antibodies directed to the LDL receptor or to other membrane receptors. We believe it to be result of the high binding affinity of the antibody for the receptor at physiological temperatures.

Such binding characteristics provided a direct quantification of the complement of cell surface versus intracellular receptors in rabbit fibroblasts in culture. If each molecule of antibody binds one receptor molecule, it follows that confluent cultures of rabbit fibroblasts after 24 h of pre-incubation in LDFCS contain a total of 309 fmol of receptor/mg of cell protein (Fig. 2) (or 37 ng/mg of cell protein, assuming a molecular mass of 120 kDa for the unreduced receptor protein) (Gherardi *et al.*, 1988b). If each molecule of McAb MAC188 binds two molecules of receptor, the total estimated pool of LDL receptors in rabbit fibroblasts is 74 ng/mg of cell protein, of which just under 50% (Fig. 1) are on the cell surface at any one time. These values are in good agreement with the results of radioimmunoassay experiments on human fibroblasts (60 ng/mg of cell protein) (Knight *et al.*, 1986b).

The behaviour of McAb MAC188 *in vivo* is of considerable interest. Based on the results of the experiments with cells in culture, we predict that administration of a high enough dose of McAb MAC188 will lead to transient receptor deficiency, and possibly hypercholesterolaemia, due to sequestering of the receptors inside the cells. Injection of a low dose of MAC188, however, resulted in only partial removal of the antibody from the circulation (Fig. 5), when it had been expected that the majority of antibody would be cleared. One explanation for the incomplete clearance after 15 min is that the antibody is heterogeneous and the fraction of antibody left in serum is inactive. Because McAb MAC188 was produced by fusion with the light-chain-secreting myeloma Y3 and both spleen and myeloma light chains are secreted (Gherardi, 1987), it is likely that the product of association of the spleen heavy chain and the myeloma light chain is inactive. Indeed, there has been a report of high-affinity binding of the heavy chain alone to a protein antigen (Ward *et al.*, 1989), but the generality of this finding remains to be established. There is the additional possibility that a proportion of the antibody pool is inactivated during iodination even though the enzyme-based iodination procedure used in our studies is very mild compared with most chemical methods. Both of these points (the effect of the light chain on receptor binding and the possible loss of activity upon iodination) require further study. It is perhaps worth noting that the presence of inactive ligand(s) is of limited importance in a variety of conventional receptor experiments in which inactive species, if present, are removed during the washing steps. The presence of such inactive species is, however, of great significance in experiments *in vivo* where they will recirculate for very long periods of time and provide a high experimental background.

An additional point which remains to be studied is the use of Fab or F(ab')₂ fragments rather than intact anti-receptor antibodies *in vivo*. The non-specific binding of intact McAb MAC188 to rabbit fibroblasts is very low (Figs. 1 and 2) and so is the binding/uptake of the control antibody MAC221 in certain tissues (such as the adrenal glands) (Table 2). However, the uptake of the control antibody in other tissues (especially the liver) is notable. It is possible that this may be due to binding of

the antibody via the Fc receptor and that Fab fragments could decrease it.

To our knowledge there has been only one prior attempt to measure the expression of the LDL receptor *in vivo* with McAbs (Huettinger *et al.*, 1984). Huettinger and colleagues injected an anti-receptor McAb (IgG 9D9) into both normal and Watanabe heritable hyperlipaemic rabbits and found that the plasma clearance and tissue uptake of the antibody were delayed in the mutant rabbit strain. Although we have not carried out a direct comparison between the two antibodies, it is clear that competition by LDL for receptor binding is higher with McAb 9D9 and that the plasma clearance of McAb 9D9 is slower (Huettinger *et al.*, 1984) (Figs. 4 and 5). It is most likely that these differences are due to different binding affinities of the two antibodies at physiological temperatures and/or different epitope specificities.

Finally, let us return to the general question of the importance of assessing the expression of the LDL receptor *in vivo* in animals and man. The first reason concerns the relationship between receptor levels in hepatic and extrahepatic cells. There are a number of studies on the levels of LDL receptors in peripheral cells (typically blood mononuclear cells), but it is not yet clear whether the LDL receptors in these cells are regulated by the same processes that set the level of expression of the liver receptor or, simply, by the concentration of LDL to which white blood cells are exposed in the circulation. There are certainly major differences in the expression of the LDL receptor in rabbit tissues (Spady *et al.*, 1987; Gherardi, 1987; the present paper, Table 2) and similar results have been obtained in all species that have been studied, including man. This would suggest that an accurate assessment of the number of functional hepatic LDL receptors involves a receptor assay on liver tissue and would strengthen the desirability of a non-invasive technique. The second reason relates to drug discovery. There is conclusive evidence for a role of the liver LDL receptors in influencing plasma LDL levels (Starzl *et al.*, 1984; Spady & Dietschy, 1985, 1988), and the potential for pharmacological control of the hepatic receptor has been clearly demonstrated experimentally with drugs such as mevinolin and cholestyramine (Slater *et al.*, 1980; Kovanen *et al.*, 1981). New drugs (perhaps acting directly rather than indirectly on the liver LDL receptor) also require adequate procedures to assess their ability to increase the number of LDL receptors in the liver *in vivo*.

With regard to patients with hypercholesterolaemia, the need for non-invasive and reliable approaches to assess the level of LDL receptor activity is equally compelling. The now classic studies of Goldstein and Brown on patients with FH have clearly identified an important group with a defect in the LDL receptor gene (Brown & Goldstein, 1986), but there is evidence that changes in receptor numbers may lead to a functional defect in other patients with hypercholesterolaemia (Grundy & Vega, 1985; Vega & Grundy, 1986). Studies *in vivo* with anti-receptor McAbs such as MAC188 may be feasible and informative in these patients.

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