Antipeptide antibody against the human low-density-lipoprotein receptor

Characterization and cross-reactivity with bovine lymphocytes

Genevieve. AGNANI,* Catherine DELPIERRE,† Soraya BENHAMAMOUCH,* Gérard TORPIER,‡ Claude CACHERA,§ Monique KOFFIGAN,* André TARTAR,†† Jean C. FRUCHART*§ and Véronique CLAVEY*§||

*SERLIA et INSERM U 279 Institut Pasteur, 1 rue du Pr. Calmette, B.P. 245, 59019 Lille Cédex,

†Chimie des Biomolécules Institut Pasteur, 1 rue du Pr. Calmette, B.P. 245, 59019 Lille Cédex,

‡Unité mixte INSERM U167-CNRS 624 Institut Pasteur, 1 rue du Pr. Calmette, B.P. 245, 59019 Lille Cédex, and

§Faculté de Pharmacie, 1 rue du Pr. Laguesse, 59045 Lille, France

A dodecapeptide corresponding to the external *N*-terminal sequence of the human low-density-lipoprotein (LDL) receptor was synthesized. Antibodies raised in rabbits against the peptide were purified and were shown to react specifically with the peptide and with human LDL receptor of fibroblasts, HeLa cells and lymphocytes using binding studies and immunoblotting. By indirect immunogold analysis, antibodies bound to the LDL receptor of human lymphocytes could be revealed as clusters. Anti-receptor peptide immunoglobulins specifically bound to the human HeLa cell's LDL receptor with a lower affinity than LDL ($K_d \times 3$). The anti-receptor peptide immunoglobulins and ^{125}I -labelled-LDL competed with each other for the LDL-receptor sites. Antibodies failed to react with lymphocytes of subjects with the homozygous form of familial hypercholesterolaemia. Cross-reactivity with the dodecapeptide of the bovine LDL receptor was limited, but this cross-reactivity was confirmed by the binding of anti-receptor peptide immunoglobulins to the LDL receptor from bovine lymphocytes.

INTRODUCTION

Studies on cultured human fibroblasts [1], HeLa cells [2] and blood lymphocytes [3] have defined a receptor-mediated pathway by which cells bind, internalize and degrade low-density lipoproteins (LDLs). The human LDL-receptor sequence determined by Yamamoto *et al.* [4] revealed that the protein possesses at least five domains.

The *N*-terminal domain (292 amino acids) is located at the external surface of the plasma membrane and is generally considered as corresponding to the binding domain of the receptor [5]. Schneider *et al.* [6] have shown that antibodies raised against a synthetic peptide corresponding to the first 16 amino acid residues of the *N*-terminus of bovine LDL receptor recognized the bovine receptor. These antibodies also cross-reacted to a significant extent with the human LDL receptor, although the corresponding *N*-terminal sequences differ in several positions in the two species.

In the present study we have synthesized the *N*-terminal dodecapeptide corresponding to the human LDL-receptor sequence. Antibodies raised against this peptide were shown to react with the LDL receptor on human fibroblasts, HeLa cells and lymphocytes. They also partially inhibit ^{125}I -LDL binding. Their cross-reactivity with bovine lymphocytes has been determined.

EXPERIMENTAL

Lipoproteins

Human LDL [density (ρ) 1.030–1.055 kg/l] and HDL₃ (ρ 1.12–1.21 kg/l) were isolated from fresh plasma of normal healthy donors by sequential ultracentrifugation [7].

Lipoprotein-deficient serum (LPDS) ($\rho > 1.25$ kg/l) was prepared from a pool of fresh serum by subjecting this material to two centrifugation steps at 100 000 *g* in a 50.2 Ti Rotor in a Beckman L.8 ultracentrifuge at 4 °C for 24 h. LDL was radiolabelled with Na ^{125}I (Amersham) by the iodine monochloride method described by MacFarlane [8]. The specific radioactivity was expressed in c.p.m. and is indicated in the Figure legends. The radioactivity counting efficiency was 60 %.

Methylated LDL was prepared by the method of Weisgraber *et al.* [9]. An antibody against LDL was raised in rabbits, and immunoglobulins were prepared by Na₂SO₄ (170 g/l) precipitation. This antibody recognized both LDL and methylated LDL.

Synthesis of the *N*-terminus peptide of the LDL receptor

The *N*-termini peptides of the human [4] and bovine [6] LDL receptor (Table 1) were synthesized by solid-phase technology as described by Merrifield [10].

Abbreviations used: LDL, low-density lipoprotein; HDL, high-density lipoprotein; FH, familial hypercholesterolaemia; FITC-IgG, fluorescein isothiocyanate-labelled IgG; ARP-Ig, anti-receptor peptide immunoglobulins; LPDS, lipoprotein-deficient serum; EDCI, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodi-imide hydrochloride; PBS, phosphate-buffered saline [0.1 M-sodium phosphate (pH 7.2)/0.15 M-NaCl]; PCA, phosphate/calcium/albumin [PBS/CaCl₂ (0.5 mM)/bovine serum albumin (BSA) (20 g/l)]; Boc, *t*-butoxycarbonyl; OBzl, benzyloxy; Tos, tosyl; acm, acetamidomethyl; DMEM, Dulbecco's modified Eagle Medium;

|| To whom correspondence and reprint requests should be sent, at the following address: SERLIA Institut Pasteur, 1 rue du Pr. Calmette, 59019 Lille Cédex, France.

Table 1. Comparative amino acid sequence of the N-terminal position of the human (H) and bovine (B) LDL receptor

H	Ala-Val- <u>Gly</u> -Asp- <u>Arg</u> -Cys- <u>Glu</u> -Arg-Asn-Glu-Phe- <u>Gln</u>
B	Ala-Val- <u>Glu</u> -Asp- <u>Asn</u> -Cys- <u>Gly</u> -Arg-Asn-Glu-Phe- <u>Glu</u>

N- α -Boc protection was used and trifunctional amino acids were protected as follows: Asp(OBzl); Arg(Tos); Cys(acm); Glu(OBzl). After deprotection and cleavage from the polymeric support by treatment with HF, the peptides were purified by gel filtration on Bio-Gel P₂ (Bio-Rad) using 5% acetic acid for elution. Reverse-phase h.p.l.c. and t.l.c. confirmed the homogeneity of the peptides, and amino acid analysis demonstrated that they had the expected amino acid composition.

Human antipeptide receptor antibodies (ARP-Ig)

The purified human peptide, covalently linked to tetanus toxin by using *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodi-imide hydrochloride (EDCI) as coupling agent via the peptide carboxy group, was injected into New Zealand White rabbits (0.4 mg of conjugated peptide, then two further injections at 2-week intervals (0.2 mg). Total immunoglobulins were obtained from the plasma of these animals by two successive Na₂SO₄ precipitations (170 g/l), and purified immunoglobulins were then isolated by immunoaffinity chromatography as follows: 10 mg of peptide was coupled to 2.7 g of dry aminoethyl-Sepharose 4B (Pharmacia) with soluble EDCI as coupling agent. The column was then equilibrated with 0.05 M-sodium phosphate buffer (pH 7.4)/0.15 M-NaCl, and the specific immunoglobulins were eluted with 3 M-NaSCN, pH 6.8.

Immunoglobulins were radiolabelled with Na¹²⁵I by the chloramine-T technique [11]. The specific radioactivity ranged between 500 and 1000 c.p.m./ng of protein and is indicated in Figure legends.

Before rabbit immunization, preimmune serum was obtained from the same rabbits and immunoglobulins were precipitated by Na₂SO₄ to be used as control. In some cases (indicated in Figure legends) preimmune immunoglobulins of different rabbits were used.

Immunoassay determinations

Immune-serum reactivity was determined by immunoassay as described elsewhere [12]. Briefly, microtitre plates (Costar, Cambridge, MA, U.S.A.) were coated with peptide [1 μ g in 100 μ l of 0.1 M-sodium phosphate buffer (pH 7.2)/0.15 M-NaCl (PBS)] overnight at 4 °C. After washing, dilutions of immune serum, immunoglobulins or preimmune rabbit serum were added to the wells and left for 2 h at 37 °C. The wells were washed, then diluted peroxidase-labelled sheep anti-rabbit antibodies [1:4000 (v/v)] (Pasteur Diagnostic) were added; the amount of peroxidase fixed on the solid phase was determined after substrate addition (H₂O₂, 3.5 mM; 1,2-phenylenediamine, 17 mM) at 492 nm.

A competitive immunoassay was developed to determine the ability of human and bovine N-terminal peptide to inhibit binding of purified ARP-Ig against human LDL-receptor peptide to adsorbed human peptide [13]. A non-specific reaction was shown with three different preparations of preimmune Ig.

Microtitre plates were coated with peptide (1 μ g in 100 μ l of PBS) overnight at 4 °C. After washing, 50 μ l of purified human ARP-Ig (19.2 μ g/ml) or preimmune immunoglobulins (19.2 μ g/ml) and 50 μ l of human or bovine N-terminal peptide at different concentrations were added to the wells and left for 2 h at 37 °C. The wells were washed, peroxidase-labelled sheep anti-rabbit antibodies were added and the amount of peroxidase fixed on the solid phase was determined.

Cell culture

Human fibroblasts (MRC5; Biomerieux) and HeLa cells (Williams) were grown in culture medium (RPMI 1640 for fibroblasts or DMEM for HeLa cells) containing 10% (v/v) fetal-calf serum. At 48 h before experiments, cells were preincubated in medium containing 10% (v/v) LPDS. All incubations were performed at 37 °C in an atmosphere of CO₂/air (1:19).

Human mononuclear cells were isolated under sterile conditions from fresh blood of normal donors or homozygous familial hypercholesterolaemic patients according to a modification [14] of the method of Boyüm [15]. Blood lymphocytes were also isolated, using the same conditions, from cow. The cells were cultured for 72 h in RPMI-1640 medium supplemented with L-glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μ g/ml), BSA (10 g/l) and human HDL₃ (300 μ g/ml) to enhance the requirement for cholesterol and so stimulate LDL-receptor binding capacity [16]. After preincubation, the cells were harvested by centrifugation (350 g, 10 min, 4 °C) and resuspended in 6 ml of ice-cold PCA working buffer before centrifugation and resuspension at a final concentration of 20 $\times$ 10⁶ cells/ml.

Monolayer binding studies

Binding studies of ¹²⁵I-labelled LDL or ¹²⁵I-labelled ARP-Ig, performed at 4 °C on nearly confluent cells as described by Goldstein *et al.* [17] for LDL gave a measure of membrane receptor binding of the lipoproteins. In order to assess the rates of internalization and degradation of the lipoproteins, assays were performed at 37 °C using the Goldstein & Brown procedure [1]. These assays were performed in triplicate. Non-specific binding was obtained by adding unlabelled LDL in excess (1 mg/ml) to the binding assay system or by the binding of ¹²⁵I-labelled preimmune immunoglobulins.

Indirect immunofluorescence analysis of the LDL receptor on lymphocytes

Lymphocyte binding assays were carried out in 750 μ l polyethylene Microfuge tubes that had been precoated with BSA (10 g/l). Incubations were conducted in an ice bath in a 4 °C cold-room to avoid interference due to endocytosis; 2 $\times$ 10⁶ cells were used for each binding test in a final volume of 100 μ l of PCA buffer.

LDL binding test. LDL in PCA buffer was added to lymphocytes at a final concentration of 25 μ g/ml.

Incubation was performed for 45 min, after which each tube was centrifuged in the 12042 rotor of a Sigma 202 MK centrifuge (10000 g, 2 min, 4 °C). The supernatant was discarded and the cell pellet rinsed with 300 μ l of PCA buffer then with 300 μ l of 100% fetal serum as described by Ho *et al.* [14]. The cells were then incubated for 45 min with rabbit immunoglobulins specific for human LDL (50 μ g/ml final concn.). There-

after the cells were washed twice with PCA buffer and centrifuged (4000 g, 5 min, 4 °C). The last 45 min incubation was performed with fluorescein-labelled anti-rabbit immunoglobulins (FITC-IgG; Pasteur Diagnostic) diluted 1:20 (v/v) with PCA buffer. After incubation, the cells were washed twice with PBS and centrifuged (4000 g, 5 min, 4 °C). The pellet was fixed by resuspension in 200 μ l of paraformaldehyde (10 g/l) in PBS and immediately used for cytofluorimetric studies.

ARP-Ig binding test. Affinity-purified ARP-Ig was incubated at appropriate concentrations (usually 5 μ g/ml) for affinity-purified ARP-Ig with cells for 45 min at 4 °C, then all washing steps and further incubations with FITC-IgG were performed as described above for the LDL binding test.

Blanks were prepared in which cells were incubated with preimmune immunoglobulins or with PCA buffer at each step, and lastly with FITC-IgG.

Fluorescence flow cytometry

Stained lymphocyte suspensions were analysed for surface fluorescence in an Orthocytofluorograf 50 H (Orthodiagnostic System Westwood TIA) equipped with argon and helium-neon lasers focused at the same point.

Two optical parameters were used to determine the lymphocyte populations: the light scattered at narrow angles (argon laser at 488 nm) and the axial light ion (helium-neon laser). Fluorescence signals corresponding to the light-scattering characteristics of lymphocytes were recorded to produce a histogram of lymphocytes counted against fluorescence intensity. The results are expressed as the percentage of lymphocytes with a fluorescence intensity above the threshold (% F).

Electrophoresis and ligand blotting

Cell extracts of fibroblasts and HeLa cells were prepared by using n-octyl β -D-glucopyranoside (40 mM) and partially purified on a DEAE-trisacryl column (IBF, Villeneuve la Garenne, France) as described by Beisiegel *et al.* [18].

For dot-blot analysis [19], 1 μ l (3 μ g) samples were spotted on nitrocellulose paper (0.45 μ m pore size; Millipore), which was treated with a solution of 10 mM-Tris/HCl, pH 7.4, containing 0.15 M-NaCl and BSA (50 mg/l) to block non-specific protein binding. The strips were incubated overnight at 4 °C in 20 mM-Tris (pH 8)/90 mM-NaCl/Tween 20 (1 g/l)/SDS (1 g/l) with LDL (20 μ g/ml), and different concentrations of anti-receptor antibody or preimmune immunoglobulins. Thereafter they were washed three times in 15 mM-Tris/0.15 M-NaCl/Tween 20 (5 g/l) for 10 min each and finally incubated overnight with peroxidase-labelled antibody against LDL or with peroxidase-labelled goat anti-rabbit immunoglobulins diluted 1:4000 (v/v) (Pasteur Diagnostics). After three washings, the enzymic reaction was started by addition of 1 mM-4-chloro-1-naphthol and 50 μ M-H₂O₂ in 20 mM-Tris/HCl (pH 8)/90 mM-NaCl.

One-dimensional electrophoresis was performed on 6 %-(w/v)-polyacrylamide slabs, containing SDS (1 g/l), by the method of Laemmli [20]. Samples of DEAE-trisacryl-purified extracts of HeLa cells or fibroblasts (0.2–0.8 mg/extract) were applied in 100 μ l of a buffer containing SDS (5 g/l), 10 % (v/v) glycerol and Bromophenol Blue (1 g/l). No reducing agent was added. Samples were incubated 30 min at room temperature,

and electrophoresis was carried out at 40 mA at 5 °C for 3 h. After electrophoresis, the proteins were transblotted electrophoretically on to nitrocellulose paper [21] and then treated as described above for the dot-blot procedure.

Indirect immunogold analysis of LDL receptor on lymphocytes

Indirect immunogold analysis was performed as described for the immunofluorescence analysis procedure, except that gold-labelled goat anti-rabbit immunoglobulins (GAR-G10; Janssen, Beerse, Belgium) were used after 1:40 (v/v) dilution with PCA buffer. The cells were then fixed in 1 % glutaraldehyde in 0.1 M-cacodylate buffer, pH 7.4, and postfixed in 1 % OsO₄ before embedding in Araldite. Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined with a Philips EM 420 electron microscope.

Other techniques

Cellular protein contents were determined by the Lowry technique [22], with BSA as standard.

RESULTS

Antipeptide receptor specificity

Immune sera and purified immunoglobulins from LDL-receptor-peptide-immunized animals were first tested by immunoenzymometry for their antipeptide reactivity. Both the human and the bovine peptides were coated on microtitre plates. As Fig. 1(a) shows, antibodies raised against the human peptide exhibited a high affinity for this antigen, but also cross-reacted to a significant extent with the bovine peptide. By contrast, non-specific binding of preimmune immunoglobulins was very low.

The specificity of human antipeptide immunoglobulins was then shown by a competitive immunoenzymometric assay. The human peptide was coated on the solid phase and both the human and bovine peptides were used as competitor (Fig. 1b). A total inhibition of the binding of human ARP-Ig to adsorbed human peptide was obtained with human peptide at a concentration of 12.5 μ M; a partial inhibition of 30 % was obtained with bovine peptide at a concentration of 50 μ M. No inhibition was observed using preimmune immunoglobulins.

Electrophoresis and ligand blotting

Extracts of HeLa cells or human fibroblasts that had been preincubated in medium with LPDS for 48 h were transferred to nitrocellulose membranes directly or after having been subjected to non-reducing SDS/polyacrylamide-gel electrophoresis: the ARP-Ig specifically bound a protein of apparent M_r 140000. A protein of lower M_r (about 72000) was also detected. This is probably a proteolytic product of the receptor that has been described by Schneider *et al.* [23] and that was retained on an IgG-C7 column [23]. A large excess of preimmune immunoglobulins (200 μ g/ml) failed to react with fibroblast and HeLa-cell extracts, and only 20 μ g of affinity-purified ARP-Ig/ml are necessary to obtain a good reactivity. This confirmed the specificity of the ARP-Ig (Fig. 2).

Binding studies at 4 °C on fibroblasts and HeLa cells

Binding curves (Fig. 3) were constructed by incubation of ¹²⁵I-labelled-ARP-Ig at 4 °C with monolayers of HeLa

Fig. 1. Immunoassay studies of ARP-Ig binding

(a) Microtitre plates were coated with $10 \mu\text{g}$ of (■, □) human or (◆, ◇) bovine *N*-terminal peptide/ml in PBS overnight at 4°C . After washing, the indicated concentrations of ARP-Ig were added (■, ◆). Non-specific binding was evaluated by adding different concentrations of human preimmune immunoglobulins (□, ◇). The values of these two curves (□ and ◇) are close and appear superimposed. (b) Microtitre plates were coated with $10 \mu\text{g}$ of human *N*-terminal peptide/ml in PBS overnight at 4°C . After washing, $50 \mu\text{l}$ of purified human ARP-Ig ($19.2 \mu\text{g/ml}$) and $50 \mu\text{l}$ of (■) or (▲) bovine *N*-terminal peptide at different concentrations were added to the wells. Similar experiments were performed with $50 \mu\text{l}$ of preimmune immunoglobulins ($19.2 \mu\text{g/ml}$) and different concentrations of (□) human or (△) bovine *N*-terminal peptide (superimposed curves). After incubation at 37°C for 2 h, unbound immunoglobulins were washed off according to the standard procedure, then anti-rabbit peroxidase-labelled sheep antibodies were added and the amount of peroxidase fixed was determined for the absorbance at 492 nm. The percentage of inhibition was calculated from the absorbance at 492 nm without (A_0) or with (A_p) peptide added and was $(1 - A_p/A_0) \times 100$.

cells. Such saturation curves were not obtained with preimmune ^{125}I -labelled-immunoglobulins; in this experiment, five different independent preparations were used. Non-specific-binding curves obtained with preimmune immunoglobulins or with an excess of LDL were nearly the same. Plateau values (i.e. saturation) were reached at $80 \mu\text{g}$ of total ARP-Ig/ml (Fig. 3a). Scatchard plots of the data obtained with purified immunoglobulins allowed us to calculate an apparent dissociation constant (K_d) of 40 nmol/l and suggested that each cell contained approx. 46×10^3 binding sites, assuming a univalent binding of the antibody. These results are the means for triplicate assays.

Fig. 2. Ligand blotting of LDL receptor from HeLa cells and fibroblasts

(a) Aliquots ($1 \mu\text{g}$) of solubilized membrane proteins of fibroblasts (a) or HeLa cells (b) were applied to the nitrocellulose membrane. Affinity-purified ARP-Ig ($20 \mu\text{g/ml}$), LDL ($20 \mu\text{g/ml}$) or preimmune immunoglobulins ($200 \mu\text{g/ml}$) were incubated overnight at 4°C and then revealed as described in the Experimental section. (b) HeLa-cell extracts ($200 \mu\text{g}$ of protein) were subjected to electrophoresis in 6% acrylamide slab gel containing 1 g of SDS/l. The proteins were transferred electrophoretically to nitrocellulose filter paper and incubated with total ARP-Ig or preimmune-Ig ($200 \mu\text{g/ml}$). After the first incubation, the samples were incubated with anti-rabbit peroxidase-labelled immunoglobulins as described in the Experimental section.

The affinity of LDL for HeLa cells under the same conditions (Fig. 3c) was $15.9 \pm 3 \text{ nmol/l}$ (assuming an M_r for apolipoprotein B of 550000), and each cell expressed 33×10^3 binding sites (Fig. 3c). Thus in HeLa cells there is about one ARP-Ig binding site for each high-affinity LDL binding site.

With fibroblasts, we obtained similar results. High specific binding was obtained with total ARP-Ig, and a plateau was also reached at $80 \mu\text{g/ml}$.

Internalization and degradation of ARP-Ig by HeLa cells

^{125}I -labelled-antibody was internalized in a similar fashion to that of LDL by HeLa cells (Fig. 4) and by

Fig. 3. Studies of binding of ARP-Ig and LDL to HeLa cells

HeLa cells that had been preincubated in medium containing 10% (v/v) LPDS for 48 h were washed before incubation for 2 h at 4 °C (a) with ^{125}I -labelled total ARP-Ig (1000 c.p.m./ng) or with ^{125}I -labelled-preimmune immunoglobulins (602 c.p.m./ng) (▲), (b) with ^{125}I -labelled affinity-purified ARP-Ig (856 c.p.m./ng) or ^{125}I -labelled preimmune immunoglobulins (300 c.p.m./ng) (▲) or with ^{125}I -labelled affinity-purified ARP-Ig plus a 1 mg/ml excess of unlabelled LDL (◆) or (c) with ^{125}I -labelled LDL (300 c.p.m./ng) (×) or with ^{125}I -labelled-LDL plus a 20-fold excess of unlabelled LDL (◆). Specific saturation binding curves were calculated as the difference between total binding of ARP-Ig and non-specific binding of

Fig. 4. Internalization of ARP-Ig by HeLa cells

HeLa cells (preincubated for 48 h in LPDS medium) were incubated for 4 h at 37 °C in LPDS to which had been added either ^{125}I -labelled total ARP-Ig (796 c.p.m./ng) (■) or ^{125}I -labelled preimmune immunoglobulins (651 c.p.m./ng) (▲). Specific internalization was calculated as the difference between total internalization and non-specific preimmune-immunoglobulin internalization (□).

fibroblasts. But the degradation of the ARP-Ig was slow compared with that of the lipoproteins, and there is a very small difference between degradation of ARP-Ig and preimmune Ig. The turnover time of ^{125}I -labelled-ARP-Ig saturating the cell receptors was about 4 h, whereas for LDL it was approximately half as long.

Competition between ARP-Ig and LDL for binding to the LDL receptor

Unlabelled ARP-Ig competed with ^{125}I -labelled-LDL for binding to the LDL receptor of HeLa cells at 4 °C. Binding of 10 μg of ^{125}I -LDL/ml was reduced by 50% with 200 μg of affinity-purified ARP-Ig/ml. No competition was found with preimmune immunoglobulins, and a weak competition was found with HDL₃ (Fig. 5a). These HDL₃ contained very small amounts of apolipoprotein E (< 1%) as measured by immunoassay, and this content could account for this weak competition.

Unlabelled LDL competed with ^{125}I -labelled ARP-Ig. Binding of 20 μg of ^{125}I -labelled ARP-Ig/ml was reduced by 50% with 3 μg of LDL/ml. Complete inhibition was achieved with 50 μg of LDL/ml; no competition

preimmune immunoglobulins (a and b) (□) or total binding of ^{125}I -labelled-LDL and non-specific binding of ^{125}I -labelled-LDL in the presence of unlabelled LDL (c) (□). Scatchard plots are shown as insets in (b) and (c). Abbreviations: bound, the amount of total binding of ^{125}I -labelled affinity-purified ARP-Ig or ^{125}I -labelled-LDL expressed in ng/mg of cell protein; bound/free, the amount of total binding of ^{125}I -labelled affinity-purified ARP-Ig or ^{125}I -labelled-LDL divided by the amount of unbound lipoproteins or immunoglobulins in the reaction mixture.

Fig. 5. Competition between ARP-Ig and LDL for binding to the LDL receptor

HeLa cells were incubated for 2 h at 4 °C with a constant concentration of (a) ^{125}I -labelled LDL (310 c.p.m./ng) at 10 $\mu\text{g/ml}$ or (b) ^{125}I -labelled affinity-purified ARP-Ig (552 c.p.m./ng) at 5 $\mu\text{g/ml}$ with various amounts of unlabelled LDL (x), unlabelled affinity-purified ARP-Ig (■), unlabelled preimmune immunoglobulin (▲) or HDL₃ (Δ).

was found with preimmune immunoglobulins (Fig. 5b) and this was the case for preimmune immunoglobulins from six different rabbits, including rabbits used for peptide immunization. Neither LDL nor ARP-Ig competed with ^{125}I -labelled preimmune immunoglobulins for non-specific binding (results not shown).

Immunogold analysis of ARP-Ig binding on lymphocytes

Lymphocytes from venous blood of normal donors were incubated for 48 h in RPMI/BSA medium supplemented with HDL₃ to deplete their cholesterol pools and produce maximal synthesis of LDL receptors [16]. Thereafter they were chilled to 4 °C and incubated for 45 min with affinity-purified ARP-Ig or with LDL. After washing, surface-bound ARP-Ig was detected by incubation with gold-labelled anti-rabbit immunoglobulins, which were revealed by electron microscopy; surface-bound LDL were detected after further incubation with rabbit anti-LDL then with the same gold-labelled anti-rabbit immunoglobulins.

With preimmune rabbit immunoglobulins used as control, no gold particles could be detected at the lymphocyte surface (results not shown). At 4 °C, some LDL and ARP-Ig became attached to the surface of the

cells. The general pattern of distribution of the gold spheres seemed to be the same for LDL and ARP-Ig (Fig. 6), and in all cases gold conjugates were detected as discrete patches associated with the cell membrane. With LDL, large aggregates were sometimes detected, probably due to aggregation of LDL, anti-LDL and gold anti-rabbit immunoglobulins.

Indirect immunofluorescence analysis of ARP-Ig binding to lymphocytes: cross-reactivity with bovine lymphocytes

Immunofluorescence analysis was performed as indicated above for the immunogold. Lymphocytes were preincubated for 72 h with BSA and HDL₃. Lymphocytes from 23 normal donors and from two homozygous subjects for FH were tested for LDL and ARP-Ig binding capacity. Normal lymphocytes can bind both LDL and ARP-Ig, the number of fluorescent cells being respectively $59.0 \pm 2.5\%$ and $39.2 \pm 2.4\%$ of the total. A very good correlation was obtained between LDL and ARP-Ig binding ($r = 0.95$). The measurement of non-specific binding of FITC-IgG to lymphocytes, when cells were incubated with PCA buffer (T_0) or with preimmune immunoglobulins (T_1) were very similar ($T_0 = 5.2 \pm 2.2$ and $T_1 = 5.0 \pm 0.2\%$).

Lymphocytes from the two FH homozygote subjects could not bind either LDL or ARP-Ig, and the number of fluorescent cells was not significantly different from the non-specific binding, T_0 .

Cross-reactivity of the ARP-Ig was tested with three different preparations of bovine lymphocytes: pre-immune immunoglobulins failed to react with bovine lymphocytes (7% of fluorescence cells), but ARP-Ig bound to lymphocytes (15% of fluorescent cells). Quantitative cross-reactivity was difficult to evaluate, because LDL binding (20%) of fluorescent cells was also lower with bovine lymphocytes.

DISCUSSION

The use of antipeptide antibodies offers the possibility of focusing the immune response on a predetermined region of a protein. In this case, the *N*-terminal peptide of LDL receptor was chosen because it was a part of a domain known to be localized at the surface of the cells [6] and therefore accessible to the external environment. The peptide was coupled to carrier proteins using carbodi-imide activation of the carboxy groups. By using this linkage, the *N*-terminus of the peptide was thus exposed in a similar manner as in the native protein. Results showed that immunization with this conjugated peptide resulted in the production of specific antibodies elicited mainly against the *N*-terminus of the LDL receptor.

The whole immunoglobulin fraction and specific immunoglobulins purified from it reacted with the peptide and with the partially purified human receptor as determined by immunoblotting techniques. When incubated with human fibroblasts, HeLa cells and lymphocytes, the antibody bound to the LDL receptor and could be revealed in clusters by indirect immunogold assay. The specificity of this reaction was confirmed by the failure of the antibody to bind to lymphocytes from two patients with the homozygous form of familial hypercholesterolaemia that lack LDL receptors. The affinity of ^{125}I -labelled affinity-purified ARP-Ig for HeLa cells was determined. Its K_d was 40×10^{-9} M, indicating that it

Fig. 6. Immunogold localization of ARP-Ig on human lymphocytes

This micrograph demonstrates the general pattern of gold labelling for human lymphocytes incubated for 45 min at 4 °C with affinity-purified ARP-Ig. Discrete patches of the marker are detected apparently randomly distributed on the cell surface (arrowheads). The bar represents 1 μ m. No gold-labelling marker was detected when human lymphocytes were incubated with preimmune immunoglobulins (results not shown).

bound about one-third as strongly as did LDL to these cells [K_D (10–16) $\times 10^{-9}$ M]. The number of binding sites (46×10^3), however, was slightly higher than for LDL, but that is not significantly different if we consider the mean obtained for 16 different experiments on different LDL ($N = 41 \times 10^3 \pm 6700$). The anti-receptor peptide immunoglobulins and 125 I-labelled LDL competed with each other for the LDL-receptor sites. It is noteworthy that there is cross-reactivity between the human and bovine *N*-terminal peptide. Our antibody raised against the human peptide not only cross-reacts at the level of the peptides themselves, but also at the level of their parent

proteins. It is difficult to quantify the cross-reactivity between human and bovine lymphocytes because the LDL binding differs. The two *N*-terminal dodecapeptides that we used differ from each other by four residues, three of which are non-conservative replacements; although the cross-reactivity is limited (Fig. 1), this observation indicates that sequence similarities between a short peptide used as an immunogen and an unrelated protein can produce misleading results. The differences of cross-reactivity observed in these Figures revealed to us that the competitive assay was the best method to evaluate the antibody affinity for one peptide.

Thus preimmune immunoglobulins bound weakly to peptides in direct immunoenzymometric assays, in solid phase, but not in the competitive assay. Similar observations can be made for cell binding (Figs. 3 and 5). The completion curve for bovine peptide (Fig. 1*b*) shows an inhibition of only 30% at a very high concentration (50 μ M), and it does not seem to present a significant concentration-dependence, with points for 10–12% inhibition spanning a concentration range of three orders of magnitude. Perhaps our oligoclonal sera contain several antibody subpopulations recognizing different aspects of the antigen.

Most of the monoclonal and polyclonal antibodies described have been raised against LDL receptor from bovine adrenal gland [24], and only one monoclonal antibody was raised against human LDL receptor [25]. Our anti-receptor antibody was also raised against the human LDL receptor. Schneider *et al.* [6] raised an antibody against the *N*-terminal bovine receptor peptide which bound to the LDL receptor of human fibroblasts and bovine adrenal cortex, but failed to compete with LDL for receptor binding. We think that the competition between our peptide antibody and LDL involves a proximity of the binding domains, but not necessarily identity. This hypothesis could explain the partial inhibition of the 125 I-labelled-LDL binding by the ARP-Ig (Fig. 5*a*), even at high concentrations (200 μ g/ml). The different behaviour of the present antibodies and those of Schneider *et al.* [6] probably derives from differences in the sequences of the peptides used for immunization; the synthetic peptide used by Schneider *et al.* [6] is larger by four amino acids and the bovine and human *N*-terminal dodecapeptides differ significantly in their sequence by the nature of four residues. These differences can induce a change in the conformation between the two peptides and then between antibodies raised against peptides. The approach that we used, if applied to peptide sequences along the length of the molecule, might enable one to study structure–function relationships within the receptor itself. The antibody could be also used to quantify in human lymphocytes the LDL-receptor binding capacity [26].

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