Washed human platelets bound radiiodinated low density lipoprotein (125I-LDL) to a class of saturable binding sites; they numbered 1,348±126 per platelet, and the dissociation constant (Kd) was 50.7±9 nM. 125I-LDL binding to platelets was reversible, and apparent equilibrium was attained within 25 minutes at 22°C and was characterized by forward and reverse rate constants of 1.47×10−4 sec−1×M−1 and 8×10−4 sec−1×M−1, respectively. Such binding was largely unaltered by temperature, divalent ions, and chelating agents. In addition, neither did receptor regulation (up or down) occur when platelets were loaded with cholesterol, nor did prostaglandin E1 (PGE1) increase the binding of 125I-LDL to platelets. On the other hand, the specific binding sites differed in the LDL receptor of nucleated cells.

Lipoproteins competed for the occupancy of LDL binding sites in platelets with the following order of potency: very low density >> intermediate density > high density subfraction 2. High density lipoprotein subfraction 3, heparin, and PGE1 had no effect on this binding. 125I-LDL binding to lymphocytes and fibroblasts and proteolytic degradation of 125I-LDL by lymphocytes was inhibited by the monoclonal antibody IgG-C7 directed against the LDL receptor to 88%, 85%, and 85% (p<0.001), respectively. However, with this monoclonal antibody, a blocking effect on neither 125I-LDL binding to platelets nor on LDL-enhanced platelet aggregation induced by ADP and collagen was found. Moreover, we confirmed the existence of LDL binding in platelets from patients with familial hypercholesterolemia. Our results indicate that human platelets bind LDL by saturable sites, which clearly differ from the "classical" LDL receptor in their binding properties, absence of receptor regulation, presence in platelets of familial hypercholesterolemia patients, and the lack of a blocking effect of IgG-C7 on LDL binding and LDL biological activity. (Arteriosclerosis and Thrombosis 1992;12:1353–1362)

KEY WORDS • low density lipoprotein receptor • platelet aggregation • binding sites • familial hypercholesterolemia

In recent years several in vitro studies have revealed that plasma lipoproteins can modulate the reactivity of isolated human blood platelets.1–3 Platelet-rich plasma (PRP) from patients with familial hypercholesterolemia (FH) has been reported to be hyperresponsive to certain aggregating stimuli4–6 and to produce increased amounts of thromboxane A2 when activated.7,8 When platelets are separated from their plasma environment and incubated with physiological and supraphysiological concentrations of isolated low density lipoprotein (LDL; 0.5–2 g of protein per liter), they become rapidly sensitized; at higher concentra-

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LDL Binding Sites on Platelets Differ From the "Classical" Receptor of Nucleated Cells

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In the present study we examined the differences between the LDL receptor of nucleated cells (fibroblasts and lymphocytes) and the LDL binding sites on platelets by evaluating the properties of LDL binding to washed human platelets. In addition, to confirm these differences we used the monoclonal antibody against the LDL receptor (immunoglobulin G [IgG] C7), which recognizes the first cysteine-rich repeat in the ligand-binding domain of the LDL receptor,\(^{19}\) to study their blocking ability on \(^{125}\)I-LDL binding to platelets and LDL platelet sensitization.

**Methods**

Sucrose and EDTA were purchased from E. Merck (Darmstadt, FRG); bovine serum albumin (BSA) and human serum albumin (HSA) were from Fluka Chemical AG (Basel, Switzerland); Iodo-Gen, cloramphenicol, prostataglandin E\(_2\) (PGE\(_2\)), cholesterol, heparin, and aprotinin were from Sigma Chemical Co. (St. Louis, Mo.); and Sephadex PD-10 columns and Ficol-Paque were from Pharmacia LKB (Uppsala, Sweden). The purified IgG\(_\beta\), murine monoclonal antibody, clone C7 (IgG\(_\beta-C7\)), directed against the first cysteine-rich repeat in the ligand-binding domain of the LDL receptor,\(^{19}\) was from Amersham Iberica (Madrid, Spain); culture media (RPMI-1640 and minimal essential medium [MEM]), fetal calf serum (FCS), penicillin, and streptomycin were from Flow Labs (Irvine, Scotland); plastic culture dishes and flasks were from Costar (Babedvedorp, The Netherlands); and Na\(^{125}\)I was from New England Nuclear (Boston, Mass.).

**Lipoprotein Preparation**

Human plasma lipoproteins (very low density lipoprotein [VLDL], d<1.006 g/ml; intermediate density lipoprotein [IDL], d=1.006–1.019 g/ml; LDL, d=1.019–1.063 g/ml; HDL\(_2\), d=1.063–1.125 g/ml; and HDL\(_3\), d=1.125–1.210 g/ml) and lipoprotein-deficient serum were pooled as previously described\(^{24}\) in a volume of buffer (0.15 M NaCl, 20 mM tris[hydroxymethyl]aminomethane [Tris], 5 mM glucose, and 1 mM EDTA; pH 7.4) equal to that of the discarded plasma and centrifuged at 900g for 6 minutes. For the binding studies, the platelet pellet was resuspended in a fast protein liquid chromatography device (Pharmacia LKB). Labeled LDL (100 \(\mu\)g) was loaded onto a Superose 6 column; equilibrated with 0.15 mM NaCl, 1 mM EDTA, and 0.5 g/l Na\(_2\)H\(_3\)PO\(_4\), pH 8; and developed at 0.5 ml/hr. Fractions (0.25 ml) were collected and counted on a gamma counter. A comparison of the chromatographic profiles of LDL before and after iodination strongly indicated that Iodo-Gen iodination itself did not cause aggregation or breakdown products of LDL.

**Platelet Isolation and Binding Studies**

Platelets were isolated from seven fresh platelet concentrates from controls provided by the blood bank of our hospital, from 40 ml citrated (sodium citrate 3.8%) blood from six healthy volunteers, and from patients with FH who have a partial (heterozygous, \(n=3\)) or total (homozygous, \(n=1\)) lack of LDL receptors on their nucleated cells.\(^{23}\) All patients gave informed consent. Processing was started in the laboratory within 2 hours of whole-blood collection, and all procedures were carried out at room temperature. The PRP from individual control subjects and patients was obtained by centrifugation at 300g for 10 minutes. Aliquots of fresh platelet concentrates and PRP were centrifuged at 200g for 10 minutes to remove any contaminating erythrocytes. Then EDTA (5.6 mM) was added to the PRP, and the platelets were sedimented at 900g for 6 minutes. In some cases and especially when the platelets were to be used for platelet aggregation studies (see below), PGE\(_2\) (5.6 \(\mu\)M) was also added to the PRP to prevent initial platelet activation. Then the platelet pellet was resuspended as previously described\(^{24}\) in a volume of buffer (0.15 M NaCl, 20 mM tris[hydroxymethyl]aminomethane [Tris], 5 mM glucose, and 1 mM EDTA; pH 7.4) equal to that of the discarded plasma and centrifuged at 900g for 6 minutes. For the binding studies, the platelet pellet was washed and centrifuged again as before, and the final volume was adjusted to give a platelet count of 10\(^{10}\) platelets per liter with incubation buffer (20 mM Tris-hydrochloride [HCl], pH 7.45, containing 0.15 M NaCl and 5 g/l BSA). No significant contaminating leukocytes (<10\(^{9}\)/l) were found in stained preparations. Washed platelets (0.1 ml) were incubated at room temperature for 25 minutes with varying protein concentrations of \(^{125}\)I-LDL (up to 1 g/l) in a total volume of 0.25 ml of incubation buffer. Nonspecific binding was defined as binding that was not displaced by a 20-fold excess of unlabeled LDL. Specific binding was defined as total binding minus nonspecific binding and in all experiments was about 75–85% of total binding. After incubation for 25 minutes, aliquots (0.2 ml) were removed, layered onto 0.8 ml of 20% sucrose in PBS buffer, pH 7.4, in Eppendorf tubes, and centrifuged at room temperature for 3 minutes at 16,000g. The platelet pellet was then recovered by
cutting the tips of the tubes and counted for radioactivity. Control incubations containing labeled lipoprotein but no platelets were similarly treated, and the application of 10³ cpm labeled LDL resulted in the recovery of less than 250 cpm in the tip of the tube after centrifugation. The effect of Ca²⁺, Mg²⁺, EDTA, and PGE₃ on ¹²⁵I-LDL binding to washed platelets was determined in a platelet suspension (10⁶ in a final volume of 0.25 ml of incubation buffer) containing the indicated divalent ions, chelating agent, or PGE₃ (100 μM) and 0.05 g/l ¹²⁵I-LDL.

The displacement of ¹²⁵I-LDL binding (0.05 g/l) in the presence of different concentrations and types of unlabeled lipoproteins, heparin, PGE₃, and other proteins was assessed and measured as described above. Dissociation constants (Kₛₐₚ) for the competing ligands (micrograms of protein per milliliter) were determined according to the method of Cheng and Prusoff. Displacement curves were also analyzed by Hill plots by following the method reported by Bennett and Yamamura.

To analyze the possible blocking effect of IgG-C7 on ¹²⁵I-LDL binding to platelets, we used the same experimental conditions described for lymphocytes (see below). Briefly, 10⁸ platelets were preincubated with different concentrations of IgG-C7 (up to 150 mg/l) for 10 minutes at room temperature before the addition of ¹²⁵I-LDL (0.05 g/l), and the specific binding was then determined.

Calculation of the bound lipoprotein was based on the specific activity of the labeled ligand, and the results were expressed as nanograms of protein bound per 10⁸ platelets. The specific binding was evaluated mathematically by Scatchard analysis to determine the number of binding sites and the dissociation constant by using the KINETIC/EBDA/LIGAND program.

Modulation of LDL Binding to Platelets After Cholesterol Enrichment

A modification of the procedure of Stuart et al was used to load platelets with cholesterol. Briefly, within 40 minutes of collection PRP was adjusted to a platelet count of 10⁹/ml with incubation buffer, and 10⁹ platelets (0.1 ml) were then incubated either with an ethanolic solution of nonlipoprotein cholesterol at a final concentration of 500 or 1,000 μg/ml in the incubation medium or with ethanol alone (control). The mixtures were incubated for 5 hours at 37°C with gelatin. Mixture of the incubation buffer every 30 minutes. After that the binding of ¹²⁵I-LDL to cholesterol-loaded and control platelets was measured as above. Platelet cholesterol was determined as previously described.

Aggregation Studies

Blood with 3.8% sodium citrate was collected from healthy volunteers who had not taken any drugs for the previous 10 days. PRP was obtained by centrifugation at 200g for 10 minutes at room temperature. EDTA (5.6 μM) and PGE₃ (5.6 μM) were added to the PRP to prevent initial platelet activation, and the PRP was further centrifuged at 900g for 6 minutes. The platelet pellet was washed as previously described in a volume of buffer (0.15 M NaCl, 20 mM Tris, 5 mM glucose, and 1 mM EDTA; pH 7.4) equal to that of the discarded plasma and centrifuged at 900g for 6 minutes. The washed platelets were resuspended at counts in the range 3–5×10¹¹/l in Tyrode's buffer (0.14 M NaCl, 2.7 mM KCl, 1 mM CaCl₂, 12 mM NaHCO₃, 0.4 mM NaH₂PO₄, 5.5 mM glucose, 3.5 mg/ml BSA, and 10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid [HEPES]; pH 7.4). Platelets that were washed and resuspended in this manner showed "swirling" as a gross indication of maintenance of discoid shape and were fully responsive to weak agonists in the presence of fibrinogen for at least 1 hour. Platelet aggregation was performed as previously described in a four-channel aggregometer (Aggrecorder II, model PA 3220, Menarini, Barcelona, Spain). Washed platelets (0.4 ml) with added fibrinogen (600 nM) were stimulated in the aggregometer cuvette at 37°C with the selected minimal concentrations of ADP (0.5–2 μM) or collagen (0.5–1 μg/ml) that produced a 30–60% or a 40–60% change in light transmittance, respectively, within 5 minutes of the addition of the stimulating agent. Platelet aggregation induced by a combination of 0.5 g/l LDL and ADP or collagen was performed by incubating platelets for 5 minutes at 37°C with LDL or with buffer, following by addition of ADP or collagen to trigger the aggregation. The blank samples included Tyrode's buffer, fibrinogen, and LDL or saline. The effect of IgG-C7 on LDL-induced platelet reactivity was assessed by preincubation of platelets with the antibody (150 mg/l) at various periods of time and temperature (10 and 30 minutes at 4°C, 22°C, and 37°C) before the addition of LDL and ADP or collagen. Platelet aggregation was determined as above.

Lymphocyte Isolation and Binding Studies

Lymphocytes from blood (40 ml) of healthy volunteers, drawn into EDTA-containing (15%) Vacutainer tubes, were isolated by a flotation gradient in Ficoll-Paque (d=1.077 g/ml). The lymphocyte and monocyte layer was collected under sterile conditions and rinsed twice with PBS buffer, pH 7.4. The lymphocyte and monocyte pellet was resuspended in a cholesterol-deficient medium (RPMI-1640 containing 10% LPDS) and incubated for 2 hours at 37°C in a 5% CO₂ incubator to sediment the cells. Sedimented nonadherent lymphocytes were incubated for an additional 67 hours in the same medium. The lymphocytes (8×10⁷ in 0.25 ml) were preincubated for a previously selected time (10 minutes) at room temperature with or without (0.35 mg/l) IgG-C7 in a final volume of 0.3 ml of a cholesterol-deficient medium. After incubation, the medium was removed by centrifugation for 10 minutes at 2,000g, and the cells were incubated again for 2 hours with 1 ml ice-cold MEM containing 10% LPDS and ¹²⁵I-LDL (0.01 g of protein per liter). Nonspecific binding was determined in the presence of a 20-fold excess of unlabeled LDL. After incubation, 0.7-ml aliquots were removed and layered onto 0.8 ml of 20% sucrose in PBS buffer, pH 7.4, and centrifuged at room temperature for 3 minutes at 16,000g; the pellet was later recovered as described for the platelet pellet.

LDL Receptor Activity in Lymphocytes

Proteolytic degradation of ¹²⁵I-LDL (0.01 g/l) by lymphocytes was determined according to Bilheimer et al. In short, we used nonadherent lymphocytes, prepared and incubated as above, in a cholesterol-deficient medium for 67 hours to induce maximum LDL receptor
activity. Two milliliters of cell suspension containing about 2×10^4/ml was incubated for 4 hours at 37°C with \(^{125}\)I-LDL (0.01 g of protein per liter). After that the medium was removed, and the cells were sedimented by centrifugation. The activity of high-affinity LDL receptors was quantified from the capacity of cells to degrade \(^{125}\)I-LDL to noniodide trichloroacetic acid–soluble material. The content of \(^{125}\)I-labeled, soluble (noniodide) material formed by the cells and released into the medium was determined by the procedure described by Bierman et al.\(^{33}\) Nonspecific binding was obtained in the presence of a 20-fold excess of unlabeled LDL.

The effect of IgG-C7 (0.35 mg/l) was determined as described for lymphocyte binding studies. Data are expressed as nanograms of \(^{125}\)I-LDL degraded in 4 hours per milligram of total cell protein.

### Fibroblast Binding Studies

Cultured human skin fibroblasts were grown and maintained in a humidified 5% CO\(_2\) incubator at 37°C in 75-cm\(^2\) flasks containing 20 ml MEM supplemented with 10% (vol/vol) FCS, penicillin (100 units/ml), streptomycin (100 \(\mu\)g/ml), 24 mM NaHCO\(_3\), 1 mM pyruvate, and 2 mM L-glutamine. For all experiments, confluent cells were detached with a trypsin-EDTA solution, and approximately 5×10\(^4\) cells were plated in 35-mm wells containing 2 ml growth medium. On day 3 the medium was replaced with fresh medium, and on day 6 confluent cells were incubated in LPDS (2 ml fresh medium containing 5% [vol/vol] LPDS instead of FCS) for 24 hours. After that the cell monolayers were placed for 30 minutes in a 4°C cold room. The medium was then removed, and the cells were incubated for 2 hours at 4°C with 1 ml of ice-cold MEM containing 10 mM HEPES, 5% LPDS, and the indicated amounts of \(^{125}\)I-LDL and native LDL. After incubation, the cells were washed three times with 3 ml of 50 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl and 2 g/l BSA and once with 3 ml of 50 mM Tris-HCl buffer, pH 7.4, containing only 150 mM NaCl. Finally, the high-affinity specific binding was measured as described by Brown and Goldstein.\(^{34}\)

Briefly, cells were lysed by incubation in 1 ml 0.5N NaOH overnight. Aliquots (0.5 ml) were removed from each dish, and both the radioactivity (by gamma counter) and the protein concentration\(^{21}\) were determined. Results are expressed as nanograms of \(^{125}\)I-LDL bound per milligram of total cell protein. The effect of IgG-C7 on \(^{125}\)I-LDL binding was determined according to Beisiegel et al.\(^{35}\) In short, confluent fibroblasts were incubated for 24 hours with medium containing 5% LPDS and were then precultured for 1 hour at 4°C with or without 0.35 mg/ml of IgG-C7. The high-affinity binding was then measured.

### Statistical Analysis

The results are expressed as mean±SEM. Differences between means were compared by Student’s t test, with \(p<0.05\) considered significant.

### Results

**Properties of \(^{125}\)I-LDL Binding to Human Platelets**

**Saturation studies.** Figure 1 shows the results of a typical experiment of \(^{125}\)I-LDL binding to human control platelets. The binding at room temperature, derived from seven independent experiments, was saturated at a protein concentration of 0.30±0.10 g/l. A Scatchard plot of the data gave a linear correlation coefficient of \(r=−0.96\) (Figure 1, inset). With an assumed molecular weight for apoB-100 of 580 kDa, the number of binding sites per platelet (mean±SEM, \(n=7\)) was 1,348±126, with a K\(_D\) of 50.7±9 nM (27.9±5 \(\mu\)g/ml).

**Kinetic studies.** The interaction of LDL with platelets was assessed at various periods of time. As shown in Figure 2A the binding was rapid, and apparent equilibrium was reached after incubation for 25 minutes at 22°C. The observed rate constant (K\(_{ob}\)) for the association reaction, obtained by measuring the slope of the plot shown in Figure 2B, was 2.8×10\(^{-3}\)×sec\(^{-1}\). As noted in Figure 2A, \(^{125}\)I-LDL is reversibly bound. Dissociation was slower, with a half-time of approximately 22.5 minutes and a rate constant (K\(_{-}\)) of 8×10\(^{-3}\)×sec\(^{-1}\) (Figure 2C). The second-order-association rate constant (K\(_{+}\)), calculated from the following equation

\[
K_{+}=\left[K_{ob}-K_{-}\right]\times[\text{\(^{125}\)I-LDL}]^{-1}
\]

where [\(^{125}\)I-LDL] is the concentration of \(^{125}\)I-LDL used, was 1.47×10\(^{4}\)×sec\(^{-1}\)×M\(^{-1}\). The ratio of the dissociation and association constants (K\(_{-}/K_{+}\)) equals the calculated equilibrium dissociation constant for \(^{125}\)I-LDL. With these values, the kinetically derived K\(_{0}\) of \(^{125}\)I-LDL was 55 nM, which agrees with that obtained (50.7±9 nM) by saturation studies (Figure 1).
Effects of temperature and cations. The specific binding curves at 4°C and 37°C were similar to those obtained at 22°C. In three independent experiments (mean±SEM), the number of binding sites (1,350±210 and 1,420±320) and the $K_D$ (48±5 nM and 53±8 nM) at 4°C and 37°C, respectively, were similar to those at room temperature (1,348±126 and 50±9 nM). The effect of divalent ions on the binding of $^{125}$I-LDL (0.05 g/l) is displayed in Table 1. Addition of EDTA (up to 20 mM), Ca$^{2+}$ (up to 2 mM), or Mg$^{2+}$ (up to 1 mM) to the incubation medium had no effect on the binding.

Effect of PGE$_2$. In the present study, we investigated the effect of PGE$_2$ on LDL binding to washed platelets. No significant differences were obtained when the binding was performed in platelet suspensions washed with PGE$_2$ (5.6 µM; results not shown). Recently, it has been demonstrated that PGE$_2$ is capable of increasing the binding of LDL to human, rat, and swine liver apoB,E receptors in a dose-dependent manner. The presence of PGE$_1$ (100 µM) in the incubation medium had no stimulatory effect on $^{125}$I-LDL binding to platelets. The $K_D$ and $B_{max}$ values (60±5 nM and 1,169±189 binding sites per platelet, respectively) were not significantly different from those obtained in controls without PGE$_1$.

Displacement studies. Competition experiments were performed to obtain information about the specificity of the recognition of the LDL binding sites in platelets. Table 2 shows that the specificity was not typical of the classical LDL receptor of nucleated cells. Unlabeled lipoproteins competed with $^{125}$I-LDL (0.05 g/l) for the occupancy of binding sites with the following order of potency: LDL (half-maximal value [IC$_{50}$] of 0.04 g/l), cholesteryl ester, and triglyceride plant lipoproteins.

### Table 1. Effect of Divalent Ions and Chelating Agent on $^{125}$I-LDL Binding to Platelets

<table>
<thead>
<tr>
<th>Addition</th>
<th>$^{125}$I-LDL bound (ng/10$^8$ platelets)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>218±21</td>
</tr>
<tr>
<td>Calcium (1 mM)</td>
<td>206±15</td>
</tr>
<tr>
<td>Calcium (2 mM)</td>
<td>211±18</td>
</tr>
<tr>
<td>Magnesium (1 mM)</td>
<td>209±19</td>
</tr>
<tr>
<td>EDTA (20 mM)</td>
<td>205±22</td>
</tr>
</tbody>
</table>

$^{125}$I-low density lipoprotein (LDL) at a final concentration of 0.05 g of protein per liter was incubated with platelet suspension (0.1 ml) in incubation buffer containing the indicated divalent ion (chloride salts) or chelating agent in a final volume of 0.25 ml. Binding was measured as described in "Methods." The results are the mean±SEM of three experiments.
TABLE 2. Inhibition of 125I-LDL Binding to Platelets by Different Classes of Lipoproteins, Other Proteins, and Heparin

<table>
<thead>
<tr>
<th>Ligand</th>
<th>K_D (g/l)</th>
<th>IgG-C7 (g/l)</th>
<th>N_I</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL</td>
<td>0.04±0.005</td>
<td>25.0±7</td>
<td>-1.04</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.15±0.03</td>
<td>62.1±11</td>
<td>-0.79</td>
</tr>
<tr>
<td>IDL</td>
<td>0.27±0.05</td>
<td>97.5±15</td>
<td>-0.66</td>
</tr>
<tr>
<td>HDL_1</td>
<td>0.60±0.12</td>
<td>204.1±21</td>
<td>-1.09</td>
</tr>
<tr>
<td>HDL_2</td>
<td>NI</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>BSA</td>
<td>NI</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>HSA</td>
<td>NI</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Heparin</td>
<td>NI</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>PGE_1</td>
<td>NI</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

LDL, low density lipoprotein; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; HDL, high density lipoprotein; BSA, bovine serum albumin; HSA, human serum albumin; PGE_1, prostaglandin E_1.

Platelets (0.1 ml) were incubated for 25 minutes at room temperature with 125I-LDL (0.05 g/l) in the presence of varying concentrations of nonradioactive ligands in a total volume of 0.25 ml of incubation buffer, and specific 125I-LDL binding was determined as described in "Methods." K_D values are the concentrations of ligands causing 50% displacement of 125I-LDL specific binding. Dissociation constants (K_D) were calculated according to the method of Cheng and Prussoff.24 "NI" indicates no inhibition of 125I-LDL binding at concentrations of 2 g of protein per liter of HDL_1, 10 g of protein per liter of BSA and HSA, 1,500,000 units of PGE_1 per liter of heparin, and 100 µM PGE_1. Results are the mean±SEM of four experiments. The Hill coefficients (N_I) were calculated by the method of Bennett et al.28

with a K_D of 25.0 µg/ml, or 45 nM); VLDL (IC_50 of 0.15 g/l, with a K_D of 62.05 µg/ml); IDL (IC_50 of 0.27 g/l, with a K_D of 97.51 µg/ml); and HDL_2 (IC_50 of 0.60 g/l, with a K_D of 204.15 µg/ml). The Hill coefficients, calculated from the slope of displacement curves analyzed by Hill plots, were 1.04, 0.79, 0.66, and 1.09 for LDL, VLDL, IDL, and HDL_2, respectively. HDL_1 (up to 2 g/l), BSA and HSA (up to 10 g/l), heparin (up to 1,500,000 units/l), and PGE_1 (up to 100 µM) had no inhibitory effect on 125I-LDL binding to platelets. On the other hand, we found a lack of effect of PGE_1 on the competition of unlabeled LDL for 125I-LDL bound to its specific binding sites on platelets. Similar values of IC_50 for unlabeled LDL in displacement experiments in the presence or absence of PGE_1 were obtained (0.054±0.003 g/l and 0.048±0.005 g/l, respectively).

Effect of Cholesterol Loading on Platelet Composition and 125I-LDL Binding to Platelets

The content of cholesterol in platelets after incubation with 500 or 1,000 µg/ml cholesterol was 2.4±0.22 or 5.2±0.41 µg per 10^9 platelets, respectively. These values were significantly different (p<0.001) from those obtained in controls without cholesterol (0.34±0.09). The results are mean±SEM of three experiments per triplicate.

It has been reported37 that in response to cholesterol enrichment, several types of cultured cells undergo a marked decrease in LDL binding (downregulation). To find out whether receptor regulation (up or down) of the platelet binding sites occurs, we studied the effect of cholesterol loading on 125I-LDL binding to platelets.

TABLE 3. Comparative Blocking Effect of Monoclonal Antibody IgG-C7 on 125I-LDL Binding to Fibroblasts, Lymphocytes, and Platelets

<table>
<thead>
<tr>
<th>Cell type</th>
<th>125I-LDL bound (ng)</th>
<th>Percent blocking effect (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without IgG-C7</td>
<td>With IgG-C7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>0.01</td>
<td>76±6</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>0.03</td>
<td>133±9</td>
</tr>
<tr>
<td>Platelets</td>
<td>0.05</td>
<td>325±16</td>
</tr>
</tbody>
</table>

IgG, immunoglobulin G; LDL, low density lipoprotein.

Fibroblasts incubated for 24 hours with 5% lipoprotein-deficient serum, sedimented nonadherent lymphocytes (8×10^6), and washed platelets (1×10^9) were prepared as described in "Methods." Cells were preincubated for 1 hour at 4°C (fibroblasts) and for 10 minutes at room temperature (lymphocytes and platelets) with or without 0.35 mg/l (fibroblasts and lymphocytes) and 5 mg/l (platelets) of IgG-C7. Then 125I-LDL was added at the indicated final concentrations, and the specific binding in each cell type was studied (see "Methods"). Specific high-affinity binding is expressed as nanograms of 125I-LDL bound per milligram of total cell protein for fibroblasts and as nanogram of 125I-LDL bound per 8×10^6 lymphocytes or 1×10^9 platelets. Values are mean±SEM of at least three separate experiments, and the range of inhibition is also indicated.

Cholesterol loading of platelets was achieved under incubation conditions that delivered sterol into platelets by a receptor-independent process. Platelets incubated with or without cholesterol presented similar LDL binding properties for B_max and K_D values, indicating that, in contrast to the classical LDL receptor, no changes in the receptor regulation occurred in platelets.

Results of competition binding assays to lymphocytes, fibroblasts, and platelets between 125I-LDL (0.01, 0.03, and 0.05 g of protein per liter) and IgG-C7 (0.35 mg/l for lymphocytes and fibroblasts and 5 mg/l for platelets) are shown in Table 3. Binding of 125I-LDL to lymphocytes, fibroblasts, and platelets was inhibited by this monoclonal antibody to 88%, 85%, and 5%, respectively (average of the blocking effect of five experiments per duplicate). Moreover, proteolytic degradation of labeled LDL (0.01 g of protein per liter) by lymphocytes was also inhibited to 85±10% (mean±SEM, n=5) by the IgG-C7. In contrast, Figure 3 shows that at concentrations up to 150 mg/l, IgG-C7 was not an effective competitor with 125I-LDL for LDL binding sites on platelets, and only 5% inhibition was obtained. In parallel, a dose-dependent inhibition was observed in the presence of unlabeled LDL.

Properties of 125I-LDL Binding to Platelets From Patients With Familial Hypercholesterolemia

To establish whether genetic abnormalities of the LDL receptor of nucleated cells perturb LDL binding to
FIGURE 3. Line plot showing inhibition of 125I-low density lipoprotein (LDL) binding to platelets by monoclonal antibody IgG-C7 and unlabeled LDL. Washed platelets (0.1 ml) were preincubated for 10 minutes at room temperature with the indicated concentration of the IgG-C7 (●). After that the cells were incubated for 25 minutes at room temperature with 125I-LDL (0.05 g of protein per liter, 2.1 Bq/ng protein) in a final volume of 0.25 ml of incubation buffer. Control values (without IgG-C7) were 210 ng of LDL protein bound per 10^8 platelets. The ability of different concentrations of unlabeled LDL (○) to compete for 125I-LDL binding to platelets is plotted (IC_50=0.04 g of protein per liter). Results are the mean ± SEM of three experiments.

Discussion

The aim of this study was to investigate possible differences between LDL binding sites on platelets and the described LDL receptor of nucleated cells. To achieve this, different approaches such as a complete characterization of binding, the use of platelets from patients with a genetic disorder for the classical LDL receptor, and the capability of LDL to enhance agonist-induced platelet aggregation were used.

Saturation studies showed that washed human platelets bound 125I-LDL to saturable binding sites, numbering about 1,348 per platelet, and the K_d was 50±9 nM. When we determined the rate constants for association (K_a), and dissociation (K_d), the equilibrium dissociation constant K_d was 55 nM. If the 125I-LDL binding reaction obeys the mass-action law, then the dissociation constants of kinetic, displacement, and saturation studies should be equal. We have found a K_d of 55 and 45 nM in kinetic and displacement experiments, respectively. These results are in reasonable agreement with the data obtained by saturation studies, so we can affirm that the 125I-LDL binding to platelets obeys the mass-action law. Furthermore, the 125I-LDL binding to platelets was rapid and reversible, and an apparent equilibrium was attained within 25 minutes at 22°C; it was unaltered by temperature and either divalent cations or chelating agents. These results were similar to those previously reported, except for one study that described that LDL binding to platelets was dependent on time and temperature. A change in receptor regulation (up or down) was observed neither when platelets were loaded with cholesterol nor when they were incubated in the presence of PGE, (100 μM). In contrast, it is well known that LDL binds to nucleated cells with a 10-fold higher affinity and remains associated over time. LDL-induced platelet aggregation in response to low concentrations of ADP or collagen. The preincubation of IgG-C7 (150 mg/l) for 10 minutes with washed platelets before the addition of LDL did not produce an inhibitory effect on the enhanced sensitivity of platelets to ADP or collagen induced by LDL. In fact, the LDL-induced platelet aggregation by ADP (1 and 2 μM) and collagen (1 μg/ml) was similar in the absence (60±7%, 81±8%, and 72±6%, respectively) or presence (65±5%, 80±5%, and 74±5%, respectively) of IgG-C7. Moreover, the interaction of IgG-C7 with LDL binding sites in platelets was assessed at various preincubation periods of time (10 and 30 minutes) and temperature (4°C, 22°C, and 37°C) before the addition of LDL and ADP or collagen. With our experimental conditions, IgG-C7 was unable to block 125I-LDL binding to platelets (Table 4).

Aggregation Studies

Table 4 shows data on the in vitro sensitivity of platelets to aggregating agents in the absence and presence of LDL (0.5 g of protein per liter). In agreement with previous reports, LDL sensitized the platelets and significantly increased the percentage of platelet aggregation in response to low concentrations of ADP or collagen. The preincubation of IgG-C7 (150 mg/l) for 10 minutes with washed platelets before the addition of LDL did not produce an inhibitory effect on the enhanced sensitivity of platelets to ADP or collagen induced by LDL. In fact, the LDL-induced platelet aggregation by ADP (1 and 2 μM) and collagen (1 μg/ml) was similar in the absence (60±7%, 81±8%, and 72±6%, respectively) or presence (65±5%, 80±5%, and 74±5%, respectively) of IgG-C7. Moreover, the interaction of IgG-C7 with LDL binding sites in platelets was assessed at various preincubation periods of time (10 and 30 minutes) and temperature (4°C, 22°C, and 37°C) before the addition of LDL and ADP or collagen. With our experimental conditions, IgG-C7 was unable to block 125I-LDL binding to platelets (Table 4).
0.275 g/l, with a $K_D$ of 97.51 $\mu$g/ml) on $^{125}$I-LDL binding, a feature that as yet has not been described in the literature. The Hill constant for IDL was $-0.66$, suggesting, as with VLDL, that negative cooperation or multiple binding sites might exist. Heparin, which avidly displaced LDL binding from nucleated cells, did not inhibit $^{125}$I-LDL binding to platelets, reinforcing the idea of a different interaction with LDL binding sites. Furthermore, we did not find that PGE$_1$ increased the competition of unlabeled LDL for $^{125}$I-LDL bound to its specific binding sites on platelets, in contrast to the results recently reported by Virgolini et al$^{36}$ for nucleated cells.

Monoclonal antibodies can be used to identify many receptors to block the binding or some other biological responses of specific ligands. We used IgG-C7 against

**Figure 4.** Saturation curves of $^{125}$I-low density lipoprotein (LDL) binding to platelets from patients with familial hypercholesterolemia (FH) and control subjects. Specific binding curves of $^{125}$I-LDL to platelets are shown for three patients with heterozygous FH (●), one patient with homozygous FH (●), and six normolipidemic control subjects (▲). Experimental procedures are described in "Methods." Each value represents the mean of triplicate determinations. The SEM never reached more than 10% of the mean. The number of binding sites per platelet and the $K_D$ of heterozygous and homozygous patients was 1,589±231 sites with a $K_D$ of 49±3 nM and 1,184 sites with a $K_D$ of 54.5 nM, respectively. These values were not significantly different from those obtained in control subjects (1,400±307 sites and a $K_D$ of 52±5 nM). The Scatchard plot of the data is shown in the inset.

**Table 4.** Effect of Monoclonal Antibody IgG-C7 on LDL Sensitization of Platelet Aggregation Induced by ADP and Collagen

<table>
<thead>
<tr>
<th>Temperature/time</th>
<th>ADP (2 $\mu$M)</th>
<th>Collagen (1 $\mu$g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>LDL</td>
</tr>
<tr>
<td>4°C 10 Minutes</td>
<td>53±5</td>
<td>77±6*</td>
</tr>
<tr>
<td>30 Minutes</td>
<td>46±3</td>
<td>68±7*</td>
</tr>
<tr>
<td>22°C 10 Minutes</td>
<td>56±6</td>
<td>81±8*</td>
</tr>
<tr>
<td>30 Minutes</td>
<td>44±5</td>
<td>73±6*</td>
</tr>
<tr>
<td>37°C 10 Minutes</td>
<td>63±3</td>
<td>92±8*</td>
</tr>
<tr>
<td>30 Minutes</td>
<td>55±6</td>
<td>81±7*</td>
</tr>
</tbody>
</table>

IgG, immunoglobulin G; LDL, low density lipoprotein. The effect of IgG-C7 (150 mg/l) on the LDL sensitization of platelet aggregation induced by ADP (2 $\mu$M) and collagen (1 $\mu$g/ml) as a function of time and temperature is shown. Platelets (10$^5$) were preincubated with IgG-C7 for the times and temperatures indicated in the table before the addition of LDL (0.5 g/l) and ADP or collagen. Values are the mean±SEM of at least four separate experiments. Significant differences compared with control, $^*p<0.05$. 

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the LDL receptor to characterize the binding sites for LDL on platelets. The ability of IgG-C7 to determine and identify the LDL receptor of human fibroblasts has been described. IgG-C7 blocked the binding, uptake, and degradation of LDL in fibroblasts and allowed recognition of the function of the first cysteine-rich repeat in the ligand-binding domain of the LDL receptor, which is to bind Ca2+ but not lipoproteins. We confirmed that 125I-LDL binding to fibroblasts was inhibited by IgG-C7, whereas in lymphocytes inhibition (not described) of 125I-LDL binding and 125I-LDL proteolytic degradation was observed. This fact confirmed the utility of this monoclonal antibody in studies of the classical LDL receptor. However, under the same experimental conditions as used for lymphocytes, we were unable to obtain a blocking effect on 125I-LDL binding to platelets by this monoclonal antibody, even at a 400-fold excess (compared with lymphocytes and fibroblasts). The properties described above for LDL binding to platelets support the notion that LDL binds to platelets by high-affinity and saturable sites, which differ from those described in nucleated cells and is probably under different genetic control.

To obtain more evidence, we studied the existence of specific and saturable LDL sites in platelets from patients with a total (homozgyote) or partial (heterozygote) defect of LDL receptors. Our results confirm that platelets from these patients bind 125I-LDL with binding characteristics similar to those obtained in platelets of healthy volunteers. This fact reinforced the existence of specific and saturable LDL sites in platelets from FH patients and agrees with those reported by others. Finally, we studied the capability of LDL to enhance ADP- and collagen-induced platelet aggregation and the possible blocking effect of IgG-C7 on this biological response. There is some evidence indicating that the interaction of LDL with its specific binding sites causes LDL-induced sensitization of platelets, probably through recognition of the positively charged arginine and lysine residues of the apoB of LDL. In agreement with other studies reporting that plasma lipoproteins can modulate the reactivity of human platelets, we observed that LDL at a protein concentration of 0.5 g/l enhanced platelet reactivity to aggregating agents, such as ADP and collagen. In parallel with this and in accordance with the results described by Beitz et al., thromboxane A2 production and ATP release were increased (results not shown). It has been suggested that an enhanced intracellular cholesterol level is responsible for increased platelet aggregation. Moreover, the role of the classical LDL receptor on the homeostasis of intracellular cholesterol is well known. With consideration of these facts, the monoclonal antibody used should completely inhibit cholesterol uptake and therefore platelet aggregation. However, under our experimental conditions we found the lack of a blocking effect by IgG-C7 on the LDL-induced agonist platelet sensitization.

In summary, the binding properties, the existence of 125I-LDL binding in platelets from FH patients, and especially the lack of a blocking effect by IgG-C7 on 125I-LDL binding and also on LDL-induced platelet reactivity support the hypothesis that human platelets bind LDL by a receptor different from the classical LDL receptor of nucleated cells.

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