

**β-VLDL Metabolism by Pigeon Macrophages**

Evidence for Two Binding Sites with Different Potentials for Promoting Cholesterol Accumulation

Steven J. Adelman and Richard W. St. Clair

Previous studies from our laboratory (J Lipid Res 1988;29:643–656) have shown that thioglycolate-elicited peritoneal macrophages from White Carneau and Show Racer pigeons, like mammalian macrophages, have on their surfaces specific receptors for acetylated low density lipoprotein (acLDL) and β-migrating very low density lipoproteins (β-VLDL). The binding kinetics of β-VLDL were complex, however, suggesting more than one binding site. The purpose of the present study was to further characterize these β-VLDL binding sites. Scatchard analysis of 125I-β-VLDL binding curves indicated at least two classes of binding sites. The first binds pigeon β-VLDL and LDL with high affinity (Kd approximately 7 μg/ml), is down-regulated by cholesterol loading, requires calcium, and is destroyed by the proteolytic enzyme, pronase. This pigeon β-VLDL receptor is specific for pigeon β-VLDL and LDL and does not recognize HDL, acLDL, methyl LDL, cynomolgus monkey LDL, or rabbit β-VLDL. Like the mammalian macrophage β-VLDL receptor, the "pigeon β-VLDL receptor" has many of the characteristics of an LDL receptor. The second class of binding sites is relatively nonspecific, recognizing both pigeon and rabbit β-VLDL, LDL, acLDL, methyl LDL, and HDL. Binding to this site is not altered by incubation of macrophages with pronase or by cholesterol loading. This binding site has low affinity for β-VLDL (Kd approximately 100 μg/ml), but high capacity. We have called this the "lipoprotein binding site," a term used by others to describe similar lipoprotein binding characteristics on a variety of cells. Not only does binding to this site promote the internalization and degradation of lipoproteins, but it may also facilitate the independent uptake of cholesterol. This conclusion is based on the observation that more cholesterol accumulates in cells incubated with rabbit β-VLDL, which binds only to the lipoprotein binding site, than can be accounted for by β-VLDL uptake and degradation. Since the lipoprotein binding site recognizes a variety of normal, as well as abnormal, lipoproteins, it would not require the generation of abnormal lipoprotein products, as must occur with the scavenger receptor, to promote the accumulation of cholesteryl esters in macrophages of atherosclerotic lesions. This, coupled with the fact that the lipoprotein binding site is not down-regulated by cholesterol loading, suggests that it could provide an alternative mechanism to the scavenger receptor pathway for the formation of foam cells. (Arteriosclerosis 9:673–683, September/October 1989)

White Carneau (WC) pigeons are susceptible to naturally occurring and cholesterol-aggravated aortic atherosclerosis, while Show Racer (SR) pigeons are resistant, even though both breeds have the same concentrations of plasma lipoproteins and other risk factors. As a result, comparative studies with these breeds of pigeons provides a unique opportunity to understand the biochemical and cellular factors that influence susceptibility to atherosclerosis at the level of the arterial wall.

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Both WC and SR pigeons form cholesteryl ester–rich β-VLDL in response to cholesterol feeding.\(^7\) In contrast to mammalian β-VLDL, no apoprotein E (apo E) can be detected in pigeon β-VLDL or in any of the other pigeon lipoproteins.\(^7\) This is of interest since a number of recent studies have demonstrated that apo E mediates the interaction of β-VLDL with β-VLDL receptors on mammalian macrophages,\(^8\) which are now believed to be modified forms of the low density lipoprotein (LDL) receptor.\(^10\)\(^11\) Thus, the ligand(s) responsible for the binding of pigeon β-VLDL to pigeon macrophages must be fundamentally different from those responsible for binding to mammalian macrophages. In addition to binding to a high-affinity site on pigeon macrophages, Scatchard analysis of \(4{°}C\) binding data suggests that pigeon β-VLDL can also interact with a second, lower-affinity site.\(^6\)

Our previous study showed that rabbit β-VLDL, a lipoprotein rich in apo E, was a poor competitor for the binding of pigeon β-VLDL to pigeon macrophages.\(^6\) This is consistent with the conclusion that apo E does not mediate the high-affinity binding of β-VLDL to pigeon macrophages. Rabbit β-VLDL was also a poor competitor for the binding of acetate low density lipoprotein (acLDL) to the scavenger receptor on these cells. Despite poor competition of rabbit β-VLDL for binding to these two high-affinity binding sites, preliminary studies demonstrated that rabbit β-VLDL was capable of stimulating substantial cholesteryl esterification and cholesteryl ester accumulation in pigeon macrophages. Based on these observations, we set out to further characterize the metabolism of rabbit β-VLDL by pigeon macrophages. The results of these studies indicate that pigeon macrophages possess a low-affinity binding site, which recognizes a variety of lipoproteins. This is similar to the lipoprotein binding site first described by Bachorik et al.\(^12\)\(^13\) on hepatocyte membranes. The results indicate that rabbit β-VLDL, which binds to this site, stimulates the accumulation of cholesteryl by a process that can only be partially explained by internalization and degradation of the intact β-VLDL.

**Methods**

Materials

Sodium \(^{125}\)I-iodide (IMS 300, carrier-free, in NaOH solution, pH 7 to 11) and \(^1\)C-oleic acid (CFA 243) were purchased from Amersham Corporation, Arlington Heights, Illinois. Silica gel–coated glass plates for thin-layer chromatography (TLC) were purchased from American Scientific Products, McGraw Park, Illinois. Stigmasteryl and oleic acid were purchased from Applied Science Laboratories, Incorporated, State College, Pennsylvania. Promega, fibrinogen, and bovine serum albumin (BSA) were from Sigma Chemical Company, St. Louis, Missouri. Eagle’s minimal essential medium (MEM), fetal bovine serum (FBS), and calf serum were obtained from Hazelton Research Products, Denver, Pennsylvania. Tissue culture dishes were purchased from Falcon Plastics, Miami, Florida. Fluid thiglycolate medium was purchased from Difco Laboratories, Detroit, Michigan and was prepared as described by the manufacturer. Gel electrophoresis supplies were from Pharmacia, Piscataway, New Jersey. All other chemicals were reagent grade and were purchased from Fisher Scientific Company, Raleigh, North Carolina.

Lipoproteins

Animals were fasted overnight before blood was obtained for lipoprotein isolation. Rabbit β-VLDL was obtained from New Zealand White rabbits (Franklin, Wake Forest, NC) who were fed a pelleted rabbit chow diet\(^6\) containing 0.5% cholesterol and 10% lard for at least 1 month. Pigeon β-VLDL and hypercholesterolemic LDL were from pigeons fed a cholesterol-rich (0.5% cholesterol, 10% lard) diet for at least 3 months. Normal pigeon LDL and high density lipoprotein (HDL) were from pigeons fed a pelleted grain diet. Monkey LDL was obtained from *Macaca fascicularis* monkeys fed Purina monkey chow to which cholesterol was added.\(^4\) Lipoproteins were isolated from the pooled plasma of approximately 12 pigeons, six monkeys, and three rabbits. β-VLDL was isolated from the plasma of hypercholesterolemic pigeons and rabbits by ultracentrifugation (\(d < 1.006 \text{ g/ml}\)) as described.\(^14\) LDL and HDL fractions were obtained by a combination of ultracentrifugation and agarose column chromatography on 4% agarose (Bio-Gel A-15m, Bio-Rad Laboratories, Richmond, CA).\(^16\) AcLDL was prepared from hyperlipemic monkey and pigeon LDL.\(^17\) Lipoproteins were iodinated by the iodine monochloride method.\(^18\) Less than 10% of the \(^{125}\)I in the lipoprotein preparations was soluble in chloroform and methanol (2:1), and more than 97% was precipitable in 10% trichloroacetic acid (TCA). All lipoproteins were stored in a sterile state and were sterilized again just before use with a 0.45-μm Millipore filter (Millipore Corporation, Bedford, MA), and they were used within 3 weeks of isolation. The chemical composition of the lipoproteins was determined as described previously.\(^7\) The specific activities of the iodinated lipoproteins ranged from 200 to 600 cpm/ng protein. Unless otherwise indicated, all lipoprotein concentrations were added to cells in culture on the basis of lipoprotein protein.

Cell Culture

WC and SR pigeons (6 to 12 months of age) were obtained from our breeding colony and were fed a cholesteryl-free pelleted grain diet. Elicited peritoneal macrophages were obtained as described by using thioglycolate.\(^18\) Experiments in which cells were incubated at \(37{°}C\) were carried out in a CO\(_2\) incubator with bicarbonate-buffered MEM containing 2.5 mg/ml lipoprotein-deficient serum (LPDS).\(^19\) Experiments at \(4{°}C\) were carried out in the same medium buffered with 4-(1-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and incubated in air.

**Determination of \(^{125}\)I-labeled Lipoprotein Binding and Metabolism**

Macrophages were incubated with \(^{125}\)I-pigment β-VLDL or \(^{125}\)I-rabbit β-VLDL at the protein concentrations indicated in the legends of the figures and tables. Cell surface binding was determined after 3 hours at \(4{°}C\), while lipoprotein uptake and degradation were measured after 5 hours at \(37{°}C\), unless indicated otherwise. Preliminary experiments showed that equilibrium of binding at \(4{°}C\) for all
were identified by comparison to authentic cholesterol and cholesteryl ester standards. 3H and 14C were counted in a Beckman LS 230 liquid scintillation counter.

**Results**

**Characteristics of Pigeon and Rabbit β-Very Low Density Lipoprotein**

The composition of representative samples of β-VLDL from cholesterol-fed pigeons and rabbits is shown in Table 1. The β-VLDLs from both species were rich in cholesteryl esters. Cholesteryl esters accounted for 58% to 60% of the mass of pigeon β-VLDL and 67% of the mass of rabbit β-VLDL. The percentages of total protein, phospholipid, and unesterified cholesterol were similar for both rabbit and pigeon β-VLDL. On the average, rabbit β-VLDL had a greater cholesterol/protein ratio than pigeon β-VLDL. The percent cholesterol ester and the cholesterol/protein ratio were positively correlated with plasma cholesterol concentrations for both pigeons and rabbits (data not shown).

The apoproteins of rabbit and WC pigeon β-VLDL are compared in Figure 1. The major apoproteins of rabbit β-VLDL are apo B (B-100 and B-48) and apo E. The major apoproteins of pigeon β-VLDL correspond in size to apo B-100 and apo A-I, with several unidentified proteins also present. In marked contrast to rabbit β-VLDL, pigeon β-VLDL contained no detectable apo E as demonstrated by this intentionally overloaded gel. The lack of apo E is a consistent finding for all pigeon lipoproteins, whether isolated from fed or fasted, normal or hypercholesterolemic, WC or SR pigeons.7

**Binding and Metabolism of Rabbit β-Very Low Density Lipoprotein by Pigeon Macrophages**

β-VLDL from cholesterol-fed rabbits binds to a receptor on mammalian macrophages that recognizes apo E.8,9

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**Table 1. Composition of Pigeon and Rabbit β-Very Low Density Lipoprotein**

<table>
<thead>
<tr>
<th>Source</th>
<th>Plasma chol (mg/dl)</th>
<th>Prot</th>
<th>PL</th>
<th>TG</th>
<th>UC</th>
<th>CE</th>
<th>Chol/Prot</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit β-VLDL (N=11)</td>
<td>2249±215</td>
<td>5.1±0.31</td>
<td>12.9±0.70</td>
<td>4.5±0.88</td>
<td>10.8±0.40</td>
<td>66.7±1.33</td>
<td>10.2±0.57</td>
</tr>
<tr>
<td>WC β-VLDL (N=6)</td>
<td>1872±376</td>
<td>5.6±0.42</td>
<td>15.4±0.43</td>
<td>5.9±1.01</td>
<td>13.0±0.25</td>
<td>60.1±1.01</td>
<td>8.8±0.73</td>
</tr>
<tr>
<td>SR β-VLDL (N=3)</td>
<td>1361±189</td>
<td>6.2±0.38</td>
<td>15.4±0.82</td>
<td>8.8±2.62</td>
<td>11.8±0.96</td>
<td>57.8±2.34</td>
<td>7.40±0.13</td>
</tr>
</tbody>
</table>

β-VLDL were isolated from the pooled plasma from two to four cholesterol-fed rabbits and three to five cholesterol-fed pigeons for each preparation. The results are the means±SEM for the indicated number of β-VLDL preparations analyzed for each species. Prot=protein, PL=phospholipid, TG=triglyceride, UC=unesterified cholesterol, CE=cholesteryl ester, Chol/Prot=total cholesterol/protein, β-VLDL=beta-migrating very low density lipoproteins.
Figure 1. Apoproteins of β-migrating very low density lipoproteins (β-VLDL) from cholesterol-fed rabbits and White Carneau pigeons. Apoproteins (apo) from the d<1.006 g/ml (β-VLDL) fraction were separated by sodium dodecyl sulfate (SDS) gradient gel (4% to 30%) electrophoresis. Forty micrograms of protein was applied to each lane of the gel. The molecular weight standards were composed of (Pharmacia LMW standards) phosphorylase b, 94 000; albumin, 67 000; ovalbumin, 43 000; carbonic anhydrase, 30 000; trypsin inhibitor, 20 100; and lactalbumin, 14 400. The location of the mammalian apolipoproteins, apo B, apo E, apo A-I, apo A-II, and apo C are shown.

Since pigeon β-VLDL lacks apo E, yet is taken up by a specific receptor-mediated process by pigeon macrophages, we determined whether apo E-rich rabbit β-VLDL would bind and be metabolized by pigeon macrophages and whether the presence of apo E would enhance its binding relative to pigeon β-VLDL. Figure 2 shows surface binding at 4°C of rabbit and pigeon β-VLDL to the same batch of pigeon macrophages. As seen in this representative experiment, rabbit β-VLDL bound to WC and SR macrophages with saturable kinetics. Scatchard analysis of the specific binding data (Figure 2 inserts) suggested that rabbit β-VLDL bound to a single class of sites of relatively low affinity, but high capacity. The apparent dissociation constant (Kd), calculated from four individual experiments similar to those described in Figure 2, was 100 μM with a range of 62 to 123 μM. A direct comparison in the same experiment with rabbit and pigeon β-VLDL demonstrated that pigeon β-VLDL bound to at least two classes of binding sites on pigeon macrophages. The initial high-affinity site, estimated from the first four points on the Scatchard curve, gave an apparent Kd of 7±4 μM (N=4). The low-affinity site, estimated from the final three points on the Scatchard curve, had similar binding kinetic to the low-affinity site that bound rabbit β-VLDL. The results with pigeon β-VLDL are consistent with our previously published studies demonstrating the curvilinear nature of the Scatchard plot.

Even though rabbit β-VLDL bound to pigeon macrophages with much lower affinity than pigeon β-VLDL, it was efficiently internalized and degraded by saturable processes in both WC and SR macrophages (Figure 3).
**Figure 3.** Concentration dependence of internalization and degradation of rabbit β-migrating very low density lipoprotein (β-VLDL) by White Carneau (WC) and Show Racer (SR) macrophages. Cells were incubated for 5 hours at 37°C with the indicated concentrations of 125I-labeled rabbit β-VLDL. The β-VLDL that was internalized (cell associated) and degraded by specific processes was calculated as the difference in the amount of cell-associated and degraded material in the absence or presence of 1 mg/ml of unlabeled rabbit β-VLDL. The results are the means of triplicate determinations at each point.

The time course of metabolism of rabbit and pigeon β-VLDL by pigeon macrophages is shown in Figure 4. The concentration of lipoproteins used in this experiment was 5 μg/ml. At this concentration, pigeon β-VLDL bound primarily to its high-affinity site, while rabbit β-VLDL bound only to the low-affinity site (Figure 2). Cell-associated β-VLDL reached a plateau after 1 to 2 hours for both rabbit and pigeon β-VLDL. Degradation of pigeon β-VLDL was linear for 12 hours, with some decrease in degradation rate by 24 hours. Degradation of rabbit β-VLDL was linear over the full 24 hours. Nearly twice as much pigeon β-VLDL was degraded as rabbit β-VLDL at the β-VLDL concentrations used in this experiment. Results shown are for WC macrophages, but similar results were obtained with SR macrophages (data not shown).

**Specificity of Binding of Rabbit β-Very Low Density Lipoprotein to Pigeon Macrophages**

Competition experiments were carried out at 4°C for 3 hours to determine the specificity of binding of rabbit β-VLDL to pigeon macrophages. Figure 5 demonstrates that a variety of lipoproteins will compete for the binding of 125I-rabbit β-VLDL. Monkey LDL and pigeon LDL from hypercholesterolemic birds, acLDL, and pigeon HDL competed to a similar extent but were not as effective as rabbit or pigeon β-VLDL. LDL from normocholesterolemic pigeons was less effective than that from hypercholesterolemic pigeons. A nonlipoprotein control protein, fibrinogen, showed no competition. These data suggest that rabbit β-VLDL binds to a rather nonspecific pigeon macrophage site that recognizes a variety of lipoproteins with a broad range of apoprotein compositions.

A pattern of competition by pigeon and rabbit β-VLDL, similar to that seen at 4°C, was seen with incubations at 37°C in which degradation was measured (Table 2). Both rabbit and pigeon β-VLDL were effective competitors for 125I-rabbit β-VLDL, reducing degradation by nearly 70%. In contrast, the degradation of 125I-pigeon β-VLDL was only slightly influenced by rabbit β-VLDL, while pigeon β-VLDL markedly reduced the degradation of pigeon 125I-β-VLDL. These data are consistent with the conclusion that rabbit β-VLDL is metabolized by pigeon macrophages by a relatively nonspecific lipoprotein binding site, whereas pigeon β-VLDL is metabolized by both a receptor specific for pigeon β-VLDL and the nonspecific binding site.

**Characteristics of Rabbit β-Very Low Density Lipoprotein Binding to Pigeon Macrophages**

The binding of rabbit β-VLDL to pigeon macrophages was evaluated for calcium requirement, cholesterol loading, sensitivity to proteolytic digestion, and to the lysosomotropic agent chloroquine (Figure 6). Binding of rabbit
added to each dish of White Carneau macrophages at a concentration of 3.16 μg/ml. Binding of 128I-labeled rabbit β-VLDL in the absence of competitor was 66.5±5.2 ng/mg cell protein (means±SD, N=6). Each point represents the percent change in binding of 128I-labeled rabbit β-VLDL from the value without added competitor. Duplicate dishes were used for each concentration of the various competitors. NLDL and HLDL refer to LDL in the absence of competitor over a concentration range of half log units from 0.316 to 316 μg/ml. Binding of 125I-labeled rabbit β-VLDL was diminished approximately 60% in the absence of calcium, suggesting some requirement for divalent cations. Degradation of rabbit β-VLDL appeared to be largely lysosomal, as seen by the 73% reduction in degradation when incubated in the presence of 50 μM chloroquine. Both of these results are similar to that seen previously with pigeon β-VLDL binding to pigeon macrophages.6 In contrast, the binding of rabbit β-VLDL was neither influenced by pretreatment of the cells with pronase nor was it inhibited by cholesterol loading.

Ability of Rabbit β- Very Low Density Lipoprotein to Deliver Cholesterol to Pigeon Macrophages

To determine whether the metabolism of rabbit β-VLDL by pigeon macrophages would result in cholesterol deliv-
Table 3. Effects of Pigeon and Rabbit \( \beta \)-Very Low Density Lipoprotein on Cholesterol Esterification and Accumulation in White Cameau and Show Racer Pigeon Macrophages

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>WC macrophages</th>
<th>SR macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CE syn EC</td>
<td>UC EC</td>
</tr>
<tr>
<td>LPDS</td>
<td>4±2 16±3</td>
<td>1±1 8±2</td>
</tr>
<tr>
<td>Pigeon ( \beta )-VLDL (50 ( \mu )g/ml)</td>
<td>76±13 23±3</td>
<td>105±8 94±10</td>
</tr>
<tr>
<td>Rabbit ( \beta )-VLDL (50 ( \mu )g/ml)</td>
<td>178±23 27±4</td>
<td>306±20 182±25</td>
</tr>
</tbody>
</table>

The results are expressed as micrograms per milliliter cell protein and are the means±SD, N=3.

Pigeon peritoneal macrophages were incubated with the indicated concentrations of rabbit or pigeon \( \beta \)-very low density lipoprotein (\( \beta \)-VLDL). After 24 hours, \( ^{14} \)C-oleate was added, and esterification to cholesterol was determined after an additional 24 hours.

To test this, we incubated WC macrophages with \( ^{125} \)I-labeled rabbit or pigeon \( \beta \)-VLDL with known cholesterol/protein ratios and measured internalization and degradation of the \( \beta \)-VLDLs at the indicated times (Figure 7). Cholesterol mass was measured by gas-liquid chromatography on extracts from the same cells. In another group of dishes, the same batch of cells prepared at the same time was incubated with either rabbit or pigeon \( \beta \)-VLDL or with LPDS alone, and cholesterol esterification was determined. The results of two separate experiments are shown in Figure 7. Both rabbit and pigeon \( \beta \)-VLDL were taken up and degraded in a time-dependent fashion (Panel A). Cell-associated \( ^{125} \)I-\( \beta \)-VLDL reached a plateau by 6 hours (the earliest time point measured), while degradation continued to increase for up to 48 hours. Cholesterol ester mass accumulation (Panel B) was markedly higher in cells incubated with rabbit \( \beta \)-VLDL. The total cholesterol/protein ratio of the rabbit and pigeon \( \beta \)-VLDL were 16.3 and 7.7, respectively, for Experiment 1 and 12.6 and 7.3 for Experiment 2.

Based on these differences in cholesterol/protein ratios, it was possible that the greater accumulation of cholesterol in cells incubated with rabbit \( \beta \)-VLDL was due simply to more cholesterol delivered to the cells for the same amount of \( \beta \)-VLDL protein metabolized. To test this, we calculated the total amount of \( ^{125} \)I-\( \beta \)-VLDL metabolized from the sum of cell-associated plus degraded \( ^{125} \)I-\( \beta \)-VLDL (Figure 7, Panel A). Assuming that the \( \beta \)-VLDL was taken up as an intact particle, and by knowing the protein/cholesterol ratio of the \( ^{125} \)I-labeled rabbit and pigeon \( \beta \)-VLDL, it was possible to calculate the amount of cholesterol that would have been delivered to the cells. This was compared with the absolute change in cellular cholesterol mass as determined by gas-liquid chromatography. As shown in Panel C, somewhat less cholesterol mass accumulated in cells incubated with pigeon \( \beta \)-VLDL than was predicted by \( ^{125} \)I-\( \beta \)-VLDL metabolism. This was particularly evident at the 36- and 48-hour time points and probably represents cholesterol that had effluxed from the cells after having been taken up at an earlier time. For the rabbit \( \beta \)-VLDL, however, consistently more cholesterol accumulated than could be accounted for by the metabolism of the \( \beta \)-VLDL. If some cholesterol efflux occurred in these cells as well, then the cholesterol mass accumulation actually represents a minimum estimate of what was delivered to the cells.

To more directly determine whether the increased accumulation of cholesterol by pigeon peritoneal macrophages incubated with rabbit \( \beta \)-VLDL was a function of the greater enrichment of the rabbit \( \beta \)-VLDL with cholesterol, we incubated pigeon macrophages with pigeon and rabbit \( \beta \)-VLDL selected to have the same cholesterol/protein ratio. The results are shown in Table 4 as the increase in cellular unesterified and esterified cholesterol content in cells incubated with rabbit or pigeon \( \beta \)-VLDL as compared with LPDS alone. Regardless of the source of macrophages (normal or hypercholesterolemic birds), and with two \( \beta \)-VLDL preparations, there was a consistently greater accumulation of cholesterol with rabbit \( \beta \)-VLDL as compared with pigeon \( \beta \)-VLDL. The excess cholesterol accumulation was entirely as cholesteryl ester. Thus, the excess cholesterol accumulation in cells incubated with rabbit \( \beta \)-VLDL cannot be fully explained by differences in cholesterol enrichment of the \( \beta \)-VLDL.

Discussion

Studies from a number of laboratories have demonstrated the presence of two distinct macrophage receptors...
that mediate the uptake of lipoproteins, the scavenger receptor, and the β-VLDL receptor. The scavenger receptor has been the most extensively studied (for a review, see reference 27). This receptor has been shown to recognize a variety of “abnormal” lipoproteins and to be present on monocyte/macrophages and peripheral macrophages from virtually every species tested,21 including pigeons.8 The β-VLDL receptor, although initially thought to be distinct from the LDL receptor,27,28 is now believed to be a form of the LDL receptor.10,11 Nevertheless, since there are some differences in the binding characteristics of β-VLDL to this receptor relative to the LDL receptor found on skin fibroblasts, and since we have no direct evidence in the pigeon that the β-VLDL receptor is a form of the LDL receptor, we will continue to refer to it as the β-VLDL receptor.

We have recently reported that pigeon peritoneal macrophages metabolize β-VLDL by what appears to be a receptor-mediated mechanism.6 Several of the characteristics of β-VLDL binding and metabolism by pigeon peritoneal macrophages are different from the mammalian β-VLDL receptor. As shown previously and extended in the present report, it is clear that there are at least two classes of binding sites on pigeon peritoneal macrophages for pigeon β-VLDL. This is most apparent from the curvilinear Scatchard plot. If the Scatchard curve is divided into its high- and low-affinity components (Figure 2), then the apparent Kd for binding and the B max can be estimated to be approximately 7 µg/ml and 200 ng/mg cell protein for the high-affinity site and 100 µg/ml and about 600 ng/mg cell protein for the low-affinity site. Binding to the high-affinity site (hereafter referred to as the pigeon β-VLDL receptor) is specific for pigeon β-VLDL and LDL. Based on competition studies, the high-affinity pigeon β-VLDL receptor does not bind acLDL, methyl LDL, mammalian LDL, HDL, or rabbit β-VLDL. Uptake by the pigeon β-VLDL receptor requires calcium and is downregulated by cholesterol loading. The receptor appears to be a protein, as seen by its susceptibility to pronase digestion. The β-VLDL taken up by this receptor is delivered to the lysosomes for degradation, since lysozyme agents, such as chloroquine, effectively inhibit degradation. Uptake by the pigeon β-VLDL receptor is effective in delivering cholesterol to macrophages and in stimulating cholesterol esterification. Thus, the pigeon β-VLDL receptor resembles the mammalian β-VLDL receptor in many of its characteristics.

There is, however, at least one major difference between the pigeon and mammalian β-VLDL receptor. Binding of β-VLDL to the mammalian β-VLDL receptor is mediated primarily by apo E,6,9 and although LDL will bind, it binds with an affinity that is approximately 100-fold less than β-VLDL. Pigeon β-VLDL, like all pigeon lipoproteins, does not contain apo E.7 As a result, apo E cannot be mediating the binding to the pigeon β-VLDL receptor. Furthermore, rabbit β-VLDL, a lipoprotein rich in apo E (Figure 1), does not compete for binding of pigeon β-VLDL to the pigeon β-VLDL receptor. These results clearly indicate that apo E is not mediating binding of pigeon β-VLDL to the β-VLDL receptor, although it does not exclude the possibility that other pigeon apolipoproteins (Figure 1) might participate
in binding. Cynomolgus monkey LDL, whose principal apoprotein is apo B, does not compete for binding to the pigeon β-VLDL receptor. Thus, the pigeon β-VLDL receptor, even though resembling the mammalian β-VLDL receptor in a number of ways, does not recognize either mammalian apo B or apo E.

Although rabbit β-VLDL does not bind to the pigeon β-VLDL receptor, Scatchard analysis indicates that it does bind to a single class of sites on pigeon peritoneal macrophages with low affinity (apparent Kd approximately 100 μg/ml), but high capacity (B max approximately 600 mg/ng cell protein). Evidence that pigeon and rabbit β-VLDL are binding to the same low-affinity site is based on their similar binding affinity and the ability of pigeon β-VLDL to compete for binding with rabbit β-VLDL. The low-affinity site appears to recognize not only rabbit and pigeon β-VLDL, but virtually all other lipoproteins tested, including LDL, HDL, and acLDL. Further evidence that the low-affinity site is distinct from the high-affinity pigeon β-VLDL receptor is the fact that binding of rabbit β-VLDL to the low-affinity site was not affected by pronase digestion, nor was binding decreased by cholesterol loading.

The characteristics of the low-affinity site are similar to what has been referred to by others by a variety of names including the "EDTA-resistant receptor" and "the lipoprotein binding site." This lipoprotein binding site has been described for a number of cells and tissues including, hepatocytes, liver membranes, and macrophages. Even pigeon smooth muscle cells and skin fibroblasts in culture that do not express LDL receptors bind significant amounts of lipoproteins to a site with similar characteristics. In pigeon smooth muscle cells and fibroblasts, binding to the lipoprotein binding site does not result in internalization of the lipoprotein. In contrast, in mammalian hepatocytes, internalization and degradation of LDL and HDL occurs after binding to the lipoprotein binding site, as it does with rabbit β-VLDL in human monocyte macrophages. Likewise, in pigeon macrophages, rabbit β-VLDL, which appears to bind only to the lipoprotein binding site, is efficiently internalized and degraded. Degradation occurs in the lysosomes, as seen from the inhibition of degradation by chloroquine.

Given the role of macrophages in the pathogenesis of atherosclerosis, it is important to know whether the lipoprotein binding site can play a role in cholesterol accumulation by these cells. As seen from the data in Figure 7 and Tables 3 and 4, rabbit β-VLDL was consistently more effective than the β-VLDL used in promoting cholesterol accumulation by pigeon peritoneal macrophages. Some of this difference could be accounted for by a greater cholesterol/protein ratio of the rabbit β-VLDL used in some of the experiments. However, even when the β-VLDL was selected to have the same cholesterol/protein ratio, or when the difference in this ratio was accounted for in the calculation of cholesterol delivery to cells, rabbit β-VLDL consistently caused greater accumulation of cholesterol in cells. This difference could not be explained by uptake of the intact β-VLDL particles and their degradation. In arriving at this conclusion, we have assumed that all of the cholesterol that entered the cells during the 48 hours of incubation remained in the cell. This is probably not true, however, particularly for the later time points, since the LPDS in the culture medium, and perhaps also the β-VLDL itself, may facilitate the efflux of some of the cholesterol that entered the cells at an earlier time. As a result, the data on cholesterol accumulation must be viewed as a minimum estimate of the amount of cholesterol entering on β-VLDL. Cells incubated with pigeon β-VLDL at a concentration of 50 μg/ml accumulated cholesterol at a rate equivalent to, or slightly less than, could be accounted for by β-VLDL metabolism. On the other hand, cells incubated with rabbit β-VLDL consistently accumulated from 30% to 50% more cholesterol than could be accounted for by β-VLDL metabolism. One possible explanation for this effect is that binding to the lipoprotein binding site on pigeon peritoneal macrophages not only results in the internalization and degradation of

<table>
<thead>
<tr>
<th>Experiment 1</th>
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<th>8.3</th>
<th>5.9</th>
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</table>

<table>
<thead>
<tr>
<th>Experiment 2</th>
<th>Pigeon β-VLDL</th>
<th>9.9</th>
<th>0</th>
<th>12.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit β-VLDL</td>
<td>10.0</td>
<td>3.4</td>
<td>74.0</td>
<td></td>
</tr>
</tbody>
</table>

The data for control cells are the means of quadruplicate incubations, while the data for pigeon and rabbit β-VLDL are the means of duplicate incubations.
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516 ARTERIOSCLEROSIS Vol 9, No 5, September/October 1989

\( \beta \)-VLDL, but also facilitates the uptake of cholesterol independent of the degradation of the entire \( \beta \)-VLDL particle. Such a phenomenon has been described for the uptake of HDL cholesterol in the liver and steroidogenic tissues, in rat hepatocytes and adrenal cells in culture, in human and rat fibroblasts, for LDL in skin fibroblasts, and for arterial smooth muscle cells. In steroidogenic tissue, the process of independent uptake of cholesterol appears secondary to binding to the so-called HDL receptor. Curiously, the HDL receptor has many of the same characteristics as the lipoprotein binding site, and they may, in fact, be one and the same. The specific nature of this binding site is uncertain, however. Some researchers have even suggested that HDL may bind to hydrophobic lipid domains on the cell surface rather than to a specific protein receptor.

Regardless of the nature of the interaction of \( \beta \)-VLDL with pigeon macrophages, a number of possible mechanisms exist for the independent uptake of cholesterol. Surface transfer of unesterified cholesterol between lipoproteins and cells, and the esterification and storage of this cholesterol as cholesteryl esters, has been described in rat hepatoma cells in culture and in arterial smooth muscle cells incubated with high concentrations of human LDL. Alternatively, independent uptake of cholesteryl esters could occur. Although such a phenomenon has been described in a variety of cells and tissues, the mechanism is unclear. Potential mechanisms include facilitated transfer of cholesteryl esters by transfer proteins secreted by macrophages or uptake and retroendocytosis of \( \beta \)-VLDL. The latter mechanism has been suggested for HDL metabolism by macrophages.

The greater ability of rabbit \( \beta \)-VLDL to deliver cholesterol to cells, as compared to pigeon \( \beta \)-VLDL, cannot be explained by differences in the cholesterol/protein ratio of these lipoproteins. This may suggest that more subtle differences in the surface conformation of rabbit and pigeon \( \beta \)-VLDL are important in mediating independent cholesterol transfer into macrophages. A more likely explanation, however, is that the difference is related to the particular binding site on the macrophage that mediates \( \beta \)-VLDL uptake. The majority of pigeon \( \beta \)-VLDL (unless very high concentrations are used) appears to be taken up and degraded by the high-affinity pigeon \( \beta \)-VLDL receptor. This process is tightly regulated by cellular cholesterol concentrations and apparently does not promote the independent uptake of cholesterol. Rabbit \( \beta \)-VLDL is not recognized by this regulated receptor but instead is taken up by the lipoprotein binding site, as is pigeon \( \beta \)-VLDL when present at high concentrations. The lipoprotein binding site promotes the uptake and degradation of the intact \( \beta \)-VLDL, as well as the independent uptake of cholesterol. Thus, the relative proportion of the \( \beta \)-VLDL taken up by the two binding sites on pigeon macrophages will likely determine the extent to which cholesterol accumulation occurs.

The results of this and previous studies have shown that pigeon peritoneal macrophages have at least three mechanisms for uptake of abnormal lipoproteins. These include: the scavenger lipoprotein receptor, the pigeon \( \beta \)-VLDL receptor, and the lipoprotein binding site. The pigeon \( \beta \)-VLDL receptor is readily down-regulated by cholesterol loading. As a result, in the atherosclerotic lesion, one would expect the lipoprotein binding site and the scavenger receptor to predominate. Given the high capacity of the lipoprotein binding site, and its ability to recognize a variety of normal and abnormal lipoproteins, it could play an important role in the formation of macrophage foam cells in atherosclerotic lesions. Such a mechanism should be particularly efficient in areas of the arterial wall where microdomains of very high concentrations of plasma lipoproteins are found. Although macrophages from both WC and SR pigeons express pigeon \( \beta \)-VLDL receptors, it remains to be seen whether differences in susceptibility to atherosclerosis between the two breeds of pigeons can be related to the function of the lipoprotein binding site. The demonstration that rabbit \( \beta \)-VLDL only binds to the low-affinity lipoprotein binding site on pigeon macrophages provides a useful new tool to further explore the function of this binding site in the pigeon model.

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Beta-VLDL metabolism by pigeon macrophages. Evidence for two binding sites with different potentials promoting cholesterol accumulation.
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