Lipoprotein metabolism by macrophages from atherosclerosis-susceptible White Carneau and resistant Show Racer pigeons

Steven J. Adelman[1] **and Richard W. St. Clair**[2]

The Arteriosclerosis Research Center, Department of Pathology, Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, NC 27103

Abstract The presence of specific receptors for the metabolism of acetylated low density lipoprotein (AcLDL) and β-migrating very low density lipoprotein (β-VLDL) was demonstrated in thioglycolate-elicited peritoneal macrophages from both atherosclerosis-susceptible White Carneau (WC) and resistant Show Racer (SR) pigeons. Macrophages from both breeds metabolized AcLDL through a single class of receptors that were similar, but not identical, to the scavenger receptors described in mammalian macrophages. Both pigeon and mammalian AcLDL bound to this receptor. At 37°C, AcLDL was internalized and degraded in the lysosomes, and cholesterol esterification and cholesteryl ester accumulation were stimulated. As in mammalian macrophages, AcLDL receptor activity was not down-regulated by cholesterol loading. In contrast, AcLDL binding was poorly competed for by fucoidin or polyinosinic acid, and the magnitude of cholesteryl ester accumulation was only about one-half of that seen with mouse peritoneal macrophages. Pigeon β-VLDL bound to both a high and a low affinity site on pigeon macrophages. Binding to the high affinity site was calcium-dependent, pronase-sensitive, and down-regulated by cholesterol loading. Cholesterol esterification and cholesteryl ester accumulation with β-VLDL were stimulated to an equal or greater extent than with AcLDL. Unlike mammalian macrophages, the pigeon β-VLDL receptor did not require apolipoprotein E, as evidenced by the lack of apoE in pigeon lipoproteins and by the failure of rabbit β-VLDL, containing apoE, to compete for binding. Pigeon LDL, but not mammalian LDL, was recognized by the pigeon β-VLDL receptor, suggesting that like the mammalian β-VLDL receptor, the pigeon β-VLDL receptor may be a form of an LDL receptor. This was an unexpected finding since pigeon fibroblasts and smooth muscle cells in culture do not express LDL receptors. Thus, pigeon macrophages have receptors for the uptake of abnormal lipoproteins that could play a role in the development of macrophage-derived foam cells that are prevalent in the early stages of atherosclerosis in this species. No quantitative or qualitative differences in these receptors, however, were identified that could account for the differences in atherosclerosis susceptibility between the WC and SR breeds.—**Adelman, S. J., and R. W. St. Clair.** Lipoprotein metabolism by macrophages from atherosclerosis-susceptible White Carneau and resistant Show Racer pigeons. *J. Lipid Res.* 1988. **29:** 643–656.

Supplementary key words atherosclerosis • lipoproteins • receptors • β-VLDL • acetyl LDL

Cholesteryl ester-rich cells, "foam cells," are a characteristic feature of naturally occurring atherosclerotic lesions of man and in both naturally occurring and experimentally induced atherosclerosis in animal models (1–3). Foam cells can be derived from both vascular smooth muscle cells and macrophages (4–8). Although little is known about how smooth muscle cell foam cells develop, cell culture studies have suggested several possible mechanisms of macrophage foam cell development (9, 10). These studies have shown that normal plasma lipoproteins, such as low density lipoproteins (LDL), are taken up poorly by cultured mammalian macrophages. In contrast, certain abnormal lipoproteins bind to cell surface receptors, and are internalized and metabolized by macrophages resulting in cholesteryl ester accumulation and the morphologic appearance of foam cells. LDL that has been chemically modified in vitro by acetylation, acetoacetylation, reaction with malondialdehyde, etc. (11–13), or modified by incubation with endothelial (14) or smooth muscle cells (15) in culture under conditions that permit oxidation, are taken up by "scavenger receptors" on macrophages. LDL-like macromolecules extracted from human atherosclerotic lesions are also avidly metabolized by scavenger receptors on macrophages as well as by other pathways (9, 16). Macrophages also possess a receptor that recognizes β-migrating, cholesteryl ester-rich, very low density lipoproteins (β-VLDL) (9, 10). β-VLDL has been demonstrated in the plasma of patients with dys-

Abbreviations: LDL, low density lipoproteins; HDL, high density lipoproteins; β-VLDL, beta-migrating very low density lipoproteins; AcLDL, acetyl LDL; WC, White Carneau; SR, Show Racer; FBS, fetal bovine serum; PBS, phosphate-buffered saline; MEM, Eagle's Minimum Essential Medium; LPDS, lipoprotein-deficient serum; HEPES, 4-(1-hydroxyethyl)-1-piperazine ethanesulfonic acid; K_d, apparent dissociation constant; GLC, gas–liquid chromatography; TLC, thin-layer chromatography.

[1]Present address: Metabolic Disorders, Wyeth-Ayerst Research, Philadelphia, PA 19101.

[2]To whom reprint requests should be addressed.

Downloaded from www.jlr.org by guest, on June 19, 2012

lipoproteinemia (Type III) (17) and in animals fed cholesterol-containing diets (10). The presence of β-VLDL has been associated with an increased risk of atherosclerosis in man and animals, including dogs, rabbits, and pigeons (18–20). The mammalian β-VLDL receptor recognizes apolipoprotein E of β-VLDL (21) and has recently been suggested to be an unusual form of the LDL receptor (22, 23). Since these receptors are either not down-regulated (scavenger receptor) or poorly down-regulated (β-VLDL receptor) with increasing cellular cholesterol accumulation, lipoproteins metabolized by macrophages via these receptor pathways can stimulate the accumulation of large amounts of cholesteryl esters in cultured cells resulting in cells with the morphologic appearance of foam cells (9). These findings have lead to the hypothesis that uptake of certain "abnormal" lipoproteins by macrophages of the arterial wall may play an important role in the pathogenesis of atherosclerosis.

White Carneau (WC) pigeons are susceptible to naturally occurring and cholesterol-aggravated aortic atherosclerosis (24). Histologically and biochemically, pigeon atherosclerotic lesions have many similarities to human atherosclerosis (25, 26), including the presence of macrophage-derived foam cells (27, 28). In contrast to WC pigeons, the Show Racer (SR) breed is resistant to aortic atherosclerosis (24) despite virtually identical concentrations of plasma lipoproteins (20). In both breeds, plasma cholesterol concentrations can increase to levels exceeding 2000 mg/dl after cholesterol feeding, with as much as 50% of this increase being accounted for by β-VLDL (20).

We have recently demonstrated that pigeon β-VLDL can promote the accumulation of cholesteryl esters in pigeon peritoneal macrophages in vitro and probably also in vivo (29). The purpose of the current study was to determine whether pigeon peritoneal macrophages possessed specific receptors for β-VLDL and if so, to characterize the properties of this receptor. This was of particular interest since pigeon β-VLDL does not contain apolipoprotein E (20), and pigeon skin fibroblasts and aortic smooth muscle cells in culture do not express LDL receptors (30). In addition, we examined pigeon peritoneal macrophages for the presence of the scavenger lipoprotein receptor. Finally, we asked whether there were differences in expression of β-VLDL and scavenger receptors on macrophages from WC and SR pigeons that might correlate with their known differences in susceptibility and resistance to atherosclerosis.

MATERIALS AND METHODS

Sodium [^{125}I]iodide (IMS 300, carrier-free, in NaOH solution, pH 7–11) and [1-^{14}C]oleic acid (CFA 243) were purchased from Amersham Corporation, Arlington Heights, IL. Silica gel-coated glass plates for thin-layer chromatography (TLC) were purchased from American Scientific Products, McGraw Park, IL. Stigmasterol and oleic acid were purchased from Applied Science Laboratories, Inc., State College, PA. Pronase, fucoidin, polyinosinic acid, chloroquine, and bovine serum albumin (fraction V) were from Sigma Chemical Company, St. Louis, MO. Eagle's minimal essential medium (MEM), fetal bovine serum (FBS), and calf serum were obtained from Hazelton Research Products, Denver, PA. Tissue culture dishes were purchased from Corning Glass Works, Corning, NY. All other tissue culture supplies were obtained from KC Biological, Lenexa, KS. Fluid thioglycolate medium was purchased from Difco Laboratories, Detroit, MI and prepared as described by the manufacturer. All other chemicals were reagent grade and purchased from Fisher Scientific Company, Raleigh, NC.

Lipoproteins

Animals were fasted overnight prior to obtaining blood for lipoprotein isolation. Pigeon β-VLDL and hypercholesterolemic LDL were obtained from pigeons that were fed a cholesterol-containing (0.5% cholesterol, 10% lard) diet for at least 3 months (29). Pigeon normolipemic LDL and high density lipoprotein (HDL) were obtained from pigeons consuming a cholesterol-free pelleted grain diet. Rabbit β-VLDL was obtained from New Zealand White Rabbits (Franklin Rabbitry, Wake Forest, NC) fed a pelleted rabbit chow diet containing 0.5% cholesterol and 10% lard for at least 1 month. Monkey LDL was obtained from *Macaca fascicularis* monkeys fed a diet containing 1.0 mg cholesterol/kcal (31). Blood was drawn from animals into tubes containing 1 mg/ml ethylenediamine tetraacetic acid (EDTA) and 0.4% 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) (Sigma Chemical Company), and kept at 4°C for subsequent procedures. Lipoproteins were isolated from the pooled plasma from 12 pigeons, 3 monkeys, and 3 rabbits. β-VLDL was isolated from hyperlipemic pigeon and rabbit plasma by ultracentrifugation at $d < 1.006$ g/ml as previously described (29). LDL and HDL fractions were obtained by a combination of ultracentrifugation and agarose column chromatography on Bio-Gel A-15m, (Bio-Rad Laboratories, Richmond, CA) (32). Acetyl LDL (AcLDL) was prepared from either hyperlipemic monkey or pigeon LDL (33). The cholesterol to protein ratios of LDL preparations for acetylation were between 2.2 and 2.7 for monkey LDL and 3.6 and 4.1 for pigeon LDL. Normal pigeon LDL was methylated by the method of Weisgraber, Innerarity, and Mahley (34). Lipoproteins were iodinated by the iodine monochloride method (35), with care taken to maintain the pH of the reaction at 10.0. For LDL, under these conditions, less than 2% of the ^{125}I in the lipoprotein preparations was soluble in chloroform–methanol 2:1, and greater that 98% was precipitable in 10% trichloroacetic acid (TCA). For β-VLDL, less than 10% was lipid-soluble and greater than 98% was TCA-precipitable. Lipoproteins were sterilized by filtration through a 0.45 μm Millipore filter (Millipore Corporation, Bedford, MA), and stored at 4°C.

Downloaded from www.jlr.org by guest, on June 19, 2012

Just prior to incubation with cells, the lipoproteins were again filtered through 0.45 μm filters to remove aggregates. Lipoproteins were used within 3 weeks of isolation except for AcLDL which was used within 8 weeks. The chemical composition of lipoproteins was determined as described previously (20). The specific activities of the iodinated lipoproteins ranged from 200 to 600 cpm/ng of protein.

Cell culture

WC and SR pigeons (6–12 months of age) were obtained from our breeding colony and were fed the cholesterol-free pelleted grain diet. They were anesthetized with Metofane (Pitman-Moore, Inc., Washington Crossing, NY) and peritoneal macrophages were elicited with thioglycolate. Forty eight hours later, the pigeons were killed by decapitation and the peritoneal exudate cells were removed and macrophages were isolated as previously described (29). Briefly, the exudate cells were incubated in tissue culture dishes for 2 hr at 37°C and the nonadherent cells were washed off and discarded. Fresh medium containing 10% FBS was added and the adherent macrophages were incubated overnight at 37°C. As described previously, greater than 95% of the adherent cells were judged to be macrophages based on their morphology and their ability to phagocytize latex beads and colloidal carbon (29). The next day, the media was removed, cells were washed three times with phosphate-buffered saline (PBS), bicarbonate-buffered MEM containing 2.5 mg/ml of lipoprotein-deficient serum (LPDS) (30) was added, and the cells were incubated for 24 hr at 37°C. Fresh medium containing the lipoproteins to be tested was added and the cells were incubated as described in the tables and figures. All 37°C incubations were carried out in a CO_2 incubator with bicarbonate-buffered Eagle's MEM containing 2.5 mg/ml LPDS. Incubations at 4°C were carried out in room air with MEM (without bicarbonate) that was buffered with 20 mM 4-(1-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES).

Resident mouse peritoneal macrophages were isolated from unstimulated 5- to 6-week-old Swiss albino mice (Harlen-Sprague Dawley, Indianapolis, IN) by peritoneal lavage following cervical dislocation. Once isolated, mouse peritoneal macrophages were treated as described for pigeon macrophages except that 10% calf serum was substituted for FBS.

Determination of ^{125}I-labeled lipoprotein binding and metabolism

Macrophages were incubated with the various lipoprotein preparations at the protein concentrations indicated in the tables and figures. Cell surface binding was determined after 3 hr at 4°C while lipoprotein uptake and degradation were measured after 5 hr at 37°C unless indicated otherwise. Preliminary experiments indicated that equilibrium of binding at 4°C for all lipoproteins was reached by 2 hr. After incubation at 37°C, an aliquot of culture medium was taken for determination of lipoprotein degradation by measurement of TCA-soluble, non-iodide ^{125}I (36). For both 37° and 4°C experiments, the cell layer was washed five times with PBS containing 2 mg/ml albumin, then three times with PBS. The cells were digested with 0.5 N NaOH for a minimum of 30 min and an aliquot was taken for determination of ^{125}I in a gamma spectrometer (Tracor 1185 autogamma system). A second aliquot was taken from each dish for protein determination using bovine serum albumin as the standard (37). Results were corrected for degraded lipoprotein in control dishes incubated without cells. Specific binding or metabolism was defined as the difference between the amount of ^{125}I-labeled lipoprotein that was bound, internalized, or degraded in the absence or presence of a 20-fold excess of unlabeled homologous lipoprotein.

Cellular cholesterol accumulation and esterification

Cells were incubated in the presence of the indicated lipoproteins and [1-^{14}C]oleate (0.17 mM oleate, sp act 9286 dpm/nmol) as described (38). Following incubation, the cell monolayers were washed and scraped from the dishes into PBS. Cells were disrupted by sonication, aliquots were taken for protein determination, and lipid was extracted by the method of Bligh and Dyer (39). Total and free cholesterol mass were determined by gas–liquid chromatography by the method of Ishikawa et al. (40), using stigmasterol as an internal standard. Esterified cholesterol mass was calculated as the difference between total and free cholesterol mass. Cholesterol esterification was determined by the incorporation of [^{14}C]oleate into cholesteryl esters as described previously (38).

RESULTS

AcLDL receptors on pigeon macrophages

The ability of macrophages from WC and SR pigeons to metabolize chemically altered lipoproteins was investigated using AcLDL. Saturable binding of AcLDL at 4°C occurred in both WC and SR macrophages (**Fig. 1**). AcLDL binding as evaluated by Scatchard analysis (inserts to Fig. 1), was consistent with a single class of receptors. The apparent dissociation constant (K_d) for binding was 13 ± 7 μg/ml (mean ± SD, n = three experiments with triplicate dishes at each point) for AcLDL binding to WC macrophages and 10 ± 6 μg/ml for SR macrophages. In addition to surface binding at 4°C, Fig. 1 demonstrates that AcLDL can be internalized and degraded with saturable kinetics at 37°C. No statistically significant differences were seen in any of the binding parameters between the breeds. The studies presented in Fig. 1 were conducted with monkey AcLDL. Preliminary experiments showed that monkey and pigeon

Downloaded from www.jlr.org by guest, on June 19, 2012

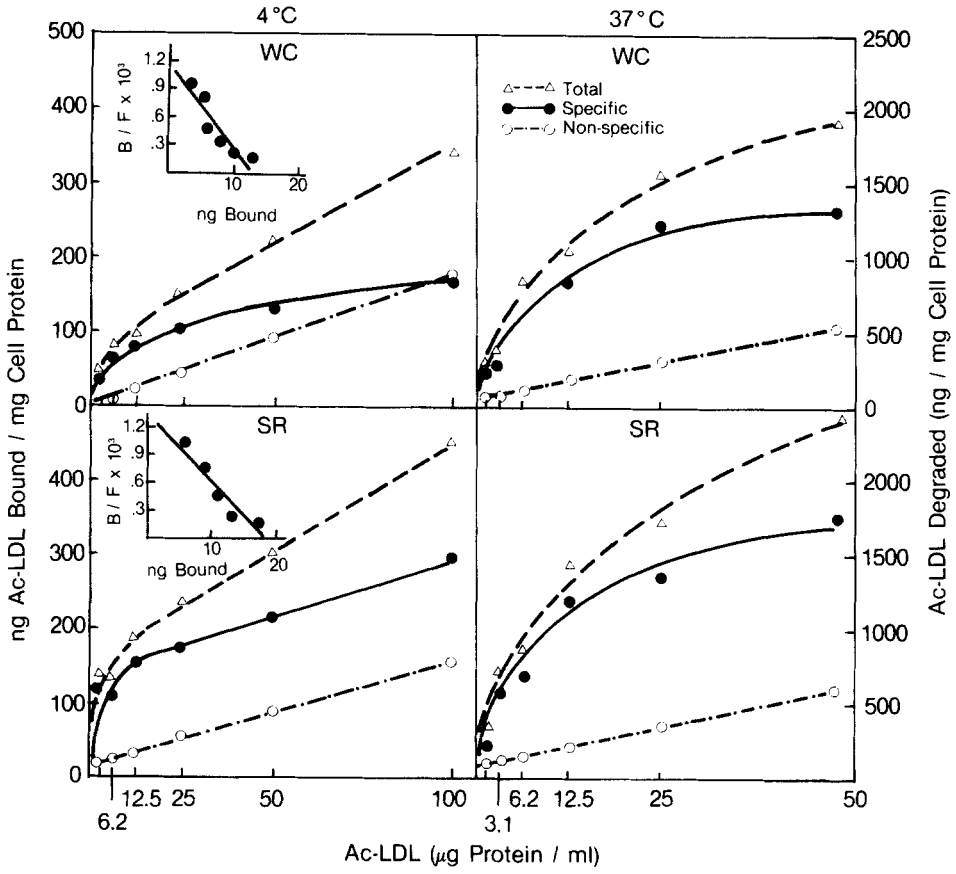

Fig. 1. Concentration dependence of binding and degradation of ^{125}I-labeled AcLDL by WC and SR macrophages. Cells were incubated with ^{125}I-labeled AcLDL prepared from cynomolgus monkey LDL, in the presence and absence of a 20-fold excess of unlabeled AcLDL. Incubations were carried out at the indicated concentrations for 3 hr at 4°C for binding or 5 hr at 37°C for degradation. Binding and degradation were determined as described in the methods section. Inserts are Scatchard plots of the 4°C specific binding data. Data are shown for two representative experiments. Each point is the mean of triplicate determinations.

AcLDL behaved similarly in their interaction with pigeon macrophages; therefore, both were used throughout this study as indicated in the tables and figures.

As shown in **Fig. 2**, AcLDL binding at 4°C was reduced by approximately 40% when calcium was removed from the culture medium. After incubation of cells with pronase for 20 min, subsequent binding of ^{125}I-labeled AcLDL was reduced 65% suggesting that the receptor is protein in nature. Chloroquine reduced the degradation of AcLDL by greater than 97% indicating that pigeon macrophages, like mammalian macrophages, transport internalized AcLDL to lysosomes for degradation. Cholesterol loading did not down-regulate the pigeon AcLDL receptor. These studies were carried out using WC pigeon macrophages. Similar results were obtained using SR pigeon macrophages (results not shown).

The specificity of ^{125}I-labeled AcLDL binding to pigeon macrophages was determined by a series of competition experiments. As shown in **Fig. 3**, binding of ^{125}I-labeled AcLDL was reduced by 80% with a 100-fold excess of unlabeled AcLDL. Although rabbit β-VLDL, and to a lesser extent pigeon β-VLDL, showed some competition for ^{125}I-labeled AcLDL, none of the other lipoproteins competed. The pigeon macrophage AcLDL receptor, unlike the mammalian AcLDL receptor (9), is only weakly competed for by fucoidin (Fig. 3, **Table 1**) or polyinosinic acid (Table 1). Although the Scatchard plot of AcLDL binding is most consistent with a single class of receptors (Fig. 1), the partial calcium dependency and the moderate degree of competition with β-VLDL suggest that there may be another less specific binding site for the uptake of AcLDL as well.

Metabolism of pigeon β-VLDL by pigeon macrophages

To determine the characteristics of the uptake of pigeon β-VLDL by pigeon macrophages, we examined the concentration dependence of β-VLDL binding and metabolism at 4°C and 37°C. **Fig. 4** demonstrates that binding and degradation of pigeon β-VLDL by macrophages from WC

Downloaded from www.jlr.org by guest, on June 19, 2012

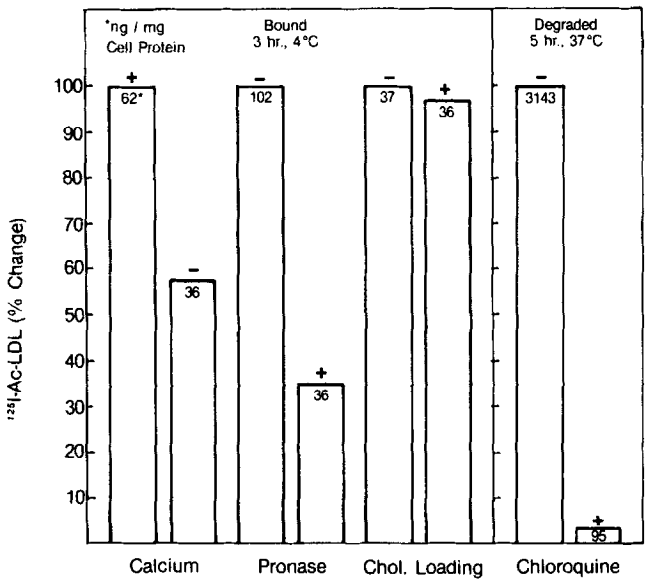

Fig. 2. Effects of calcium, pronase, chloroquine, and cholesterol loading on the AcLDL receptor pathway in WC pigeon macrophages. The amount of ^{125}I-labeled AcLDL bound and degraded is shown within each bar (ng/mg of cell protein) and the percent change is shown by the height of the bar. Cells were incubated with 5 μg/ml of ^{125}I-labeled AcLDL prepared from cynomolgus monkey LDL, in the presence or absence of 100 μg/ml of unlabeled AcLDL. Specific binding was measured after a 3-hr incubation at 4°C as described in the methods section. Specific degradation was determined after incubation of cells at 37°C for 5 hr. For calcium dependence, cells were cooled to 4°C for 1 hr and washed three times with cold calcium-free PBS containing 100 μM EDTA. ^{125}I-labeled AcLDL was added to medium containing 100 μM EDTA with or without 2 mM calcium and incubated with the cells at 4°C. For pronase sensitivity, cell monolayers were washed three times with PBS at 37°C. Serum-free MEM with or without 3 μg/ml pronase was added and the cells were incubated at 37°C. After 20 min, the cells were chilled on ice for 1 hr and washed three times with cold PBS. Fresh MEM (4°C) containing 2.5 mg/ml LPDS plus ^{125}I-labeled AcLDL ± unlabeled AcLDL was added and binding was determined after 3 hr. Chloroquine sensitivity of degradation was determined after pretreatment of cells for 1 hr at 37°C with 50 μM chloroquine. ^{125}I-Labeled AcLDL ± unlabeled AcLDL, was added to the dishes in the presence of 50 μM chloroquine and incubated for 5 hr at 37°C. Degradation was determined as described in the methods section. The effect of cholesterol loading on AcLDL binding was determined after preincubation of cells with pigeon β-VLDL (50 μg of protein/ml) for 48 hr. The cells were washed three times with PBS and incubated with MEM containing 2.5 mg/ml of LPDS for 2 hr at 37°C in order to allow internalization of surface-bound β-VLDL. The cells were then chilled at 4°C for 1 hr, washed three times with PBS, and incubated with ^{125}I-labeled AcLDL ± unlabeled AcLDL, at 4°C for 3 hr to determine binding. Cellular cholesterol content was determined in parallel dishes as described in the methods section. Total cholesterol content increased from 22 μg/mg of cell protein prior to loading to 114 μg/mg of cell protein after loading. Results are the means of triplicate determinations.

and SR pigeons is saturable, consistent with a receptor-mediated process. As demonstrated by the curvilinear Scatchard plot of the 4°C specific binding data (Fig. 4, insert), pigeon β-VLDL bound to pigeon macrophages with both high affinity and low affinity interactions. The apparent dissociation constant (K_d), approximated from the high affinity component of the Scatchard curves (first four points), averaged 7 ± 5 μg/ml (means ± SD, n = four experiments with duplicate dishes at each point) for WC macrophages and 10 ± 6 μg/ml for SR macrophages. These differences were not statistically significant. We also compared the interaction of β-VLDL from WC and SR pigeons with macrophages in order to determine whether there were differences in the β-VLDL from the two breeds. Previous studies have demonstrated that the composition of β-VLDL from WC and SR pigeons is remarkably similar (20). As shown in **Fig. 5**, there was little difference in specific binding of β-VLDL from WC and SR pigeons to either WC or SR macrophages.

The influence of calcium, pronase, chloroquine, and cholesterol loading on the binding and metabolism of pigeon β-VLDL to WC pigeon macrophages is shown in **Fig. 6.** β-VLDL binding was reduced approximately 80% when calcium was removed from the incubation medium. A similar reduction in β-VLDL binding occurred when the cells were pretreated for 20 min with pronase. In contrast to our findings with AcLDL, preloading of pigeon macrophages with cholesterol, by exposure of the cells to pigeon AcLDL, inhibited specific binding of ^{125}I-labeled pigeon β-VLDL by more than 70%. Degradation of pigeon β-VLDL at 37°C was inhibited by more than 80% with chloroquine, suggesting that degradation was mediated by lysosomal enzymes. Although the data shown in Fig. 6 are for WC macrophages, similar results were obtained using SR macrophages (data not shown).

The specificity of binding of pigeon β-VLDL to pigeon macrophages was evaluated by competition with unlabeled lipoproteins. **Fig. 7** demonstrates that binding of ^{125}I-labeled pigeon β-VLDL is inhibited by over 80% by excess unlabeled pigeon β-VLDL. In addition, both normal LDL and LDL isolated from hypercholesterolemic pigeons competed for binding of β-VLDL, with hypercholesterolemic LDL being nearly as effective as β-VLDL. Normal LDL, on the other hand, was less effective as a competitor. Previous studies have demonstrated that these three lipoproteins have similar, although not identical, apoprotein compositions, being rich in apoB-100 and apoA-I, but devoid of apoE (20). Rabbit β-VLDL competed poorly for ^{125}I-labeled pigeon β-VLDL binding, with a maximum inhibition of only 25%. Monkey LDL failed to compete, as did AcLDL and pigeon HDL. These data are consistent with the presence of a specific receptor on pigeon macrophages that recognizes pigeon β-VLDL and LDL.

Metabolism of pigeon LDL by pigeon macrophages

The competition studies with pigeon β-VLDL shown in Fig. 7 suggested that pigeon LDL could also bind to pigeon macrophages at the same site as β-VLDL. To assess LDL binding directly, we examined the concentration dependence of binding and metabolism by pigeon macrophages of LDL from normal and hypercholesterolemic pigeons. **Fig. 8** demonstrates that WC pigeon macrophages bind both normal and hypercholesterolemic pigeon LDL with saturable kinetics. No differences were seen in any of the binding parameters between macrophages or LDL from the two

Downloaded from www.jlr.org by guest, on June 19, 2012

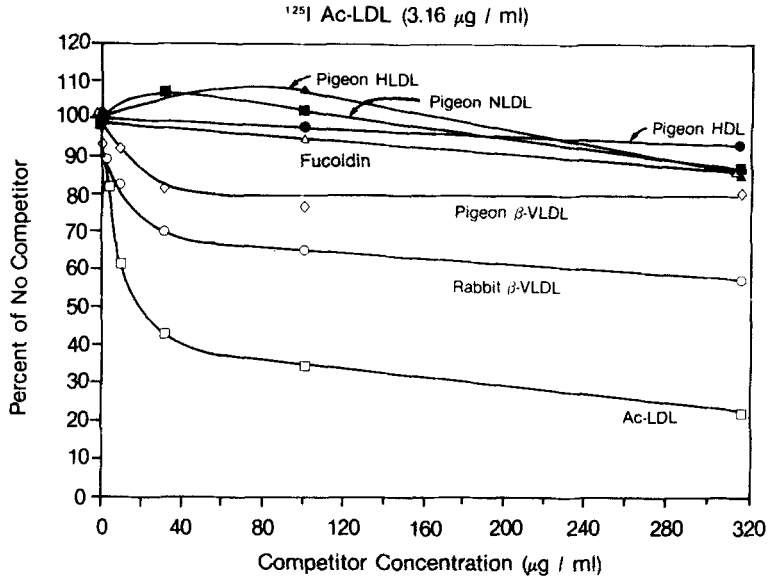

Fig. 3. Competition for binding of ^{125}I-labeled AcLDL to WC macrophages at 4°C. ^{125}I-Labeled AcLDL (3.16 μg/ml), prepared from cynomolgus monkey LDL, and the indicated competitors at a concentration range of 0.316 to 316 μg/ml were added to the cells. Binding in the absence of competitor is shown as 100% and averaged 84.4 ± 5.4 ng of ^{125}I-labeled AcLDL bound/mg of cell protein: mean ± SD, n = 6. Duplicate dishes were used at each concentration of competitor.

breeds, thus only the WC data are presented. Fig. 8 also demonstrates that, in addition to binding to surface receptors on pigeon macrophages, pigeon LDL is degraded when incubations are carried out at 37°C. Degradation was saturable, as was binding. There was less LDL protein bound and degraded with hypercholesterolemic LDL as compared to normal LDL. A similar phenomenon has been described with normal and hypercholesterolemic LDL from nonhuman primates (41).

The competition for binding and metabolism of ^{125}I-labeled normal and hypercholesterolemic LDL to WC macrophages is shown in **Fig. 9.** The pattern was similar to that shown for β-VLDL (Fig. 7). Pigeon LDL and β-VLDL were effective competitors for uptake of normal and hypercholesterolemic LDL, while monkey LDL, rabbit β-VLDL, AcLDL, and pigeon methyl LDL competed poorly. Chloroquine reduced the degradation of pigeon LDL by 97% (data not shown). Together, these data further support the conclusion that pigeon LDL and β-VLDL bind to the same site on pigeon macrophages.

Time course of lipoprotein metabolism

The time course of metabolism of AcLDL, pigeon β-VLDL, and pigeon LDL by macrophages from the two breeds of pigeons is shown in **Fig. 10.** Internalization of AcLDL occurred rapidly as indicated by the achievement of a steady state in the cell-associated radioactivity by 1 hr. Thereafter, degradation proceeded at a linear rate for at least 24 hr. Pigeon β-VLDL and LDL were also rapidly internalized with the cell-associated radioactivity plateauing by 1 hr. Degradation was linear for the first 8–12 hr, but unlike AcLDL the rate decreased thereafter. This is consistent with the ability of the β-VLDL receptor to be down-regulated. No consistent differences in the rate of metabolism of the lipoproteins were seen in cells from the two breeds of pigeons.

Cholesterol delivery to pigeon macrophages

To determine whether binding and metabolism of lipoproteins resulted in delivery of cholesterol to pigeon macrophages, we examined the effect of AcLDL, β-VLDL, and LDL on cholesterol esterification and accumulation in WC

TABLE 1. Competition of binding of AcLDL to macrophages

Competitor	WC Pigeon	Mouse
	ng bound/mg cell protein	
None	58.3 ± 5.8 (100%)	109 ± 7.7 (100%)
Pigeon AcLDL 50 μg/ml	20.0 ± 4.2 (34%)	15.0 ± 1.5 (14%)
Polyinosinic acid 632 μg/ml	40.4 ± 1.7 (70%)	26.4 ± 1.5 (15%)
Fucoidin 632 μg/ml	39.5 ± 3.7 (68%)	16.1 ± 1.4 (15%)

Cells were incubated with 3.16 μg/ml ^{125}I-labeled pigeon AcLDL plus the indicated competitors for 3 hr at 4°C. Values are mean ± SD, n = 3. Values in parentheses are the percent change from no competitor.

Downloaded from www.jlr.org by guest, on June 19, 2012

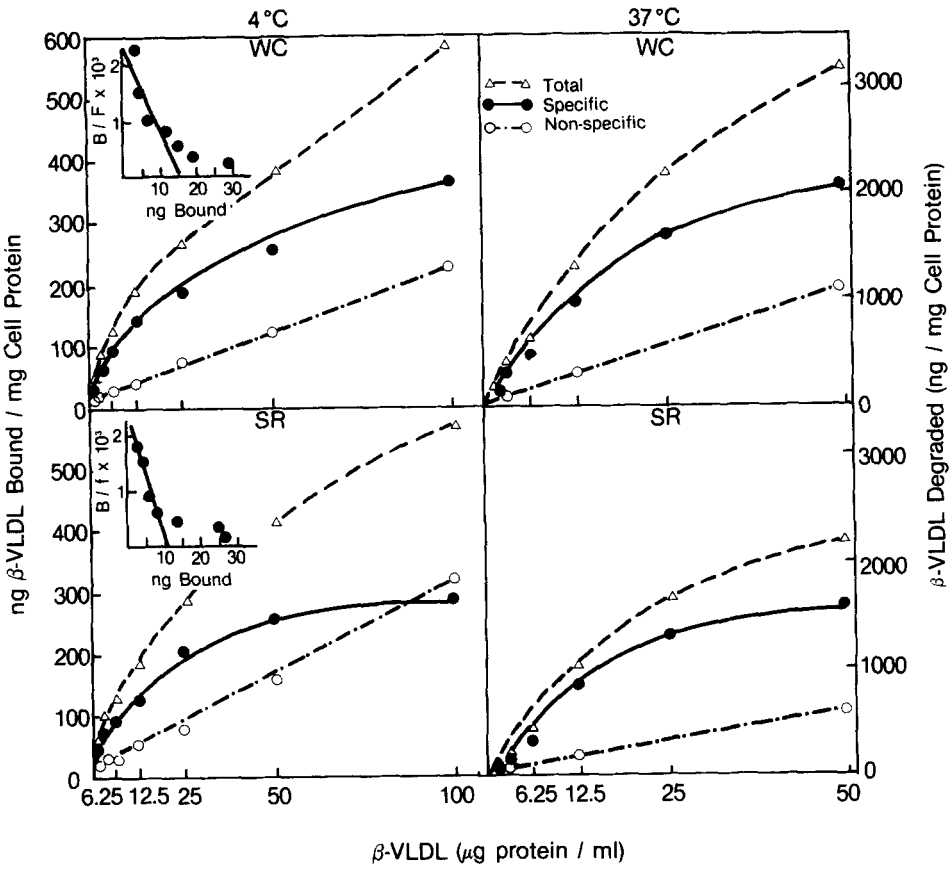

Fig. 4. Concentration dependence of binding and degradation of ^{125}I-labeled pigeon β-VLDL by WC and SR macrophages. Cells were incubated for 3 hr at 4°C or 5 hr at 37°C with ^{125}I-labeled β-VLDL, isolated from cholesterol-fed WC pigeons, in the presence and absence of a 20-fold excess of unlabeled β-VLDL. Binding and degradation were determined as described in the methods section. Inserts show the Scatchard plot of the 4°C specific binding data. The best fit linear regression line using only the first four points is shown. Data shown are from two representative experiments. Each point is the mean of triplicate determinations.

and SR macrophages (**Table 2**). Pigeon β-VLDL and hypercholesterolemic LDL and monkey and pigeon AcLDL were all effective in stimulating cholesterol esterification and cholesteryl ester accumulation. Normal pigeon LDL, however, did not stimulate either cholesterol esterification or accumulation. Although considerable variability was seen among experiments in cholesterol content and in the extent of cholesterol accumulation and esterification, there were no consistent differences between WC and SR macrophages in these parameters when exposed to the same lipoproteins.

DISCUSSION

These studies have demonstrated for the first time that thioglycolate-elicited macrophages from atherosclerosis-susceptible and resistant breeds of pigeons express receptors for AcLDL and for β-VLDL and LDL.

Pigeon macrophages metabolize AcLDL by a saturable and specific process with many similarities to the scavenger receptor pathway described in mammalian macrophages (9–11). AcLDL uptake and metabolism is not competed for by LDL or β-VLDL, and is not down-regulated by cholesterol loading. The receptor is likely protein in nature as seen by its inactivation after incubation with the proteolytic enzyme pronase. Following uptake by the AcLDL receptor, lipoproteins are directed to the lysosomes, as evidenced by the nearly complete inhibition of degradation by the lysosomotropic agent chloroquine. In contrast to mammalian macrophages (9) the polyanions fucoidin and polyinosinic acid competed poorly for AcLDL binding to pigeon macrophages. This is similar to proteoglycan-LDL complexes (42) and certain apoB-containing lipoprotein extracts from human atherosclerotic lesions (43) that bind to a receptor on mammalian macrophages with many of the characteristics of the scavenger receptor (as seen by the

Downloaded from www.jlr.org by guest, on June 19, 2012

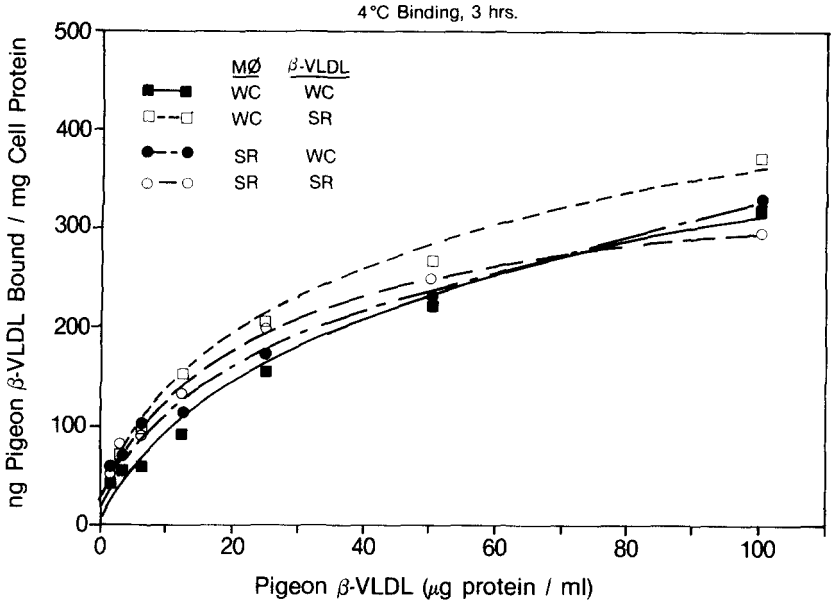

Fig. 5. Concentration dependence of binding of ^{125}I-labeled β-VLDL from WC and SR pigeons to macrophages (MØ) from the two breeds of pigeons. Thioglycolate-elicited peritoneal macrophages were isolated from WC and SR pigeons and incubated at 4°C for 3 hr with the indicated concentrations of ^{125}I-labeled β-VLDL in the presence and absence of a 20-fold excess of unlabeled homologous β-VLDL. The β-VLDL were isolated from cholesterol-fed WC and SR pigeons. The data shown are specific binding. Results are the means of triplicate determinations at each point.

ability of AcLDL to block uptake) but differ with respect to the failure of polyanions to efficiently block uptake. This suggests that some properties of the AcLDL receptor on pigeon macrophages are different than for the mammalian AcLDL receptor. Binding of AcLDL was also partially inhibited (42%) in the absence of Ca^{2+}, whereas little requirement for Ca^{2+} has been seen with mammalian macrophages (8). Finally, a potentially important difference in the AcLDL receptor pathway of pigeon macrophages, relative to mammalian macrophages, is in the extent to which cholesteryl ester accumulation occurs. In contrast to the enormous increases in cholesteryl ester content that occur in mouse peritoneal macrophages incubated for equivalent lengths of time with AcLDL [values up to and exceeding 300 μg/mg cell protein have been reported (9)], the increase in the cholesteryl ester content of the pigeon macrophages was consistently more modest, and approximately equivalent to what was seen for β-VLDL. The reason for these differences is unclear.

Pigeon β-VLDL was taken up by a saturable and specific process that was distinct from the AcLDL receptor. Binding required Ca^{2+}, was inhibited by preincubation of the cells with the proteolytic enzyme pronase, and was almost completely down-regulated by cholesterol loading of the cells. This latter property is different from AcLDL binding that was unaffected by cholesterol loading. Further evidence that this is a distinct receptor is seen from the competition data, where binding of β-VLDL was not competed for by AcLDL. The receptor on pigeon macrophages that binds β-VLDL was not entirely specific for β-VLDL, however, since both normal and hypercholesterolemic pigeon LDL competed nearly as well as β-VLDL. At 37°C β-VLDL is efficiently internalized by pigeon macrophages. Once β-VLDL is internalized, the intracellular processes appear similar to those described for mammalian macrophages. This includes processing by the lysosomes, stimulation of cholesterol esterification, and accumulation of cholesteryl esters. In fact, pigeon β-VLDL was at least as effective as AcLDL in promoting cholesteryl ester accumulation in pigeon macrophages even though there was down-regulation of the β-VLDL receptor with cholesterol accumulation. This is a potentially important difference between pigeon and mammalian macrophages that could play a role in mediating some of the differences in the pathologic features of atherosclerotic lesions of different animal models. β-VLDL appears to bind to more than a single class of binding sites on pigeon macrophages as seen from the curvilinear Scatchard plot. It is possible that at least one of these additional binding sites may represent a nonspecific lipoprotein receptor, similar to the EDTA-resistant lipoprotein receptor demonstrated in liver membranes (44). A similar nonspecific lipoprotein binding site has been described in pigeon smooth muscle cells in culture (30), where this receptor binds a variety of lipoproteins but is

Downloaded from www.jlr.org by guest, on June 19, 2012

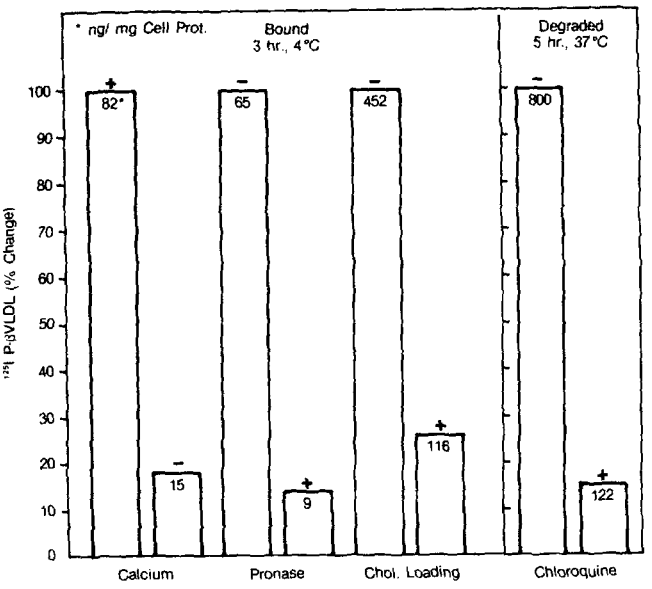

Fig. 6. Effects of calcium, pronase, chloroquine, and cholesterol loading on the β-VLDL receptor pathway in WC macrophages. The absolute amounts of ^{125}I-labeled pigeon β-VLDL (^{125}I P-β-VLDL) bound or degraded are shown within each bar (ng/mg of cell protein), and the percent change is shown by the height of the bar. Cells were incubated with 5 μg/ml of ^{125}I-labeled β-VLDL, isolated from WC pigeons, in the presence or absence of 100 μg/ml of unlabeled β-VLDL. Specific binding was measured after 3 hr incubation at 4°C as described in the methods section. Specific degradation was determined after incubation of cells at 37°C for 5 hr. Effects of calcium, pronase, and chloroquine were carried out identically to that described in Fig. 2, except that ^{125}I-labeled β-VLDL was used. The effect of cholesterol loading on the β-VLDL receptor was determined after preincubation of the cells with monkey AcLDL (200 μg/ml) for 48 hr. The remainder of the protocol was identical with the cholesterol loading experiment described in Fig. 2. Total cholesterol content increased from 20 μg/mg of cell protein prior to loading to 46 μg/mg of cell protein after loading. Results are the means of triplicate determinations.

not internalized. The extent to which this low affinity site contributes to cholesteryl ester accumulation in pigeon macrophages is unclear.

The apolipoprotein compositions of pigeon LDL and β-VLDL are similar, being rich in apoB-100 and apoA-I (20). There is, however, no evidence of a protein with a size similar to mammalian apoE in any of the lipoproteins from either normal or hypercholesterolemic WC or SR pigeons (20). This suggests, in contrast to the mammalian β-VLDL receptor where apoE is the major protein determinant required for binding (21), that the pigeon β-VLDL receptor must require different apoproteins for recognition. Furthermore, mammalian apoE and apoB, when present, do not appear to act as ligands for binding to the pigeon β-VLDL receptor, as evidenced by the fact that rabbit β-VLDL, a lipoprotein rich in apoE and apoB, failed to compete for binding to the pigeon β-VLDL receptor. Further evidence that mammalian apoB is not recognized by the pigeon β-VLDL receptor comes from the lack of competition by cynomolgus monkey LDL, an LDL that contains apoB-100 as the predominant apoprotein (45). In contrast, pigeon LDL (30, 46) and β-VLDL (Adelman, S. J., and R. W. St. Clair, unpublished observation) bind to LDL receptors on mammalian skin fibroblasts and smooth muscle cells with nearly as high affinity as mammalian LDL.

The studies described in this report have not identified the specific apoprotein(s) responsible for binding of pigeon β-VLDL to its macrophage receptor, although certain apoproteins can be eliminated. Since all pigeon lipoproteins, including β-VLDL, lack an apoprotein the size of apoE (20), other apoproteins must mediate binding to macrophages. The possibility that apoA-I can substitute in birds for apoE for such functions as reverse cholesterol transport has been suggested by Rajavashisth et al. (47). Thus, it is possible that apoA-I could also function to mediate binding to the β-VLDL receptor in birds, as apoE does in mammals. Although pigeon β-VLDL and LDL contain significant amounts of apoA-I, apoA-I does not appear to be a determinant for binding to the pigeon β-VLDL receptor since pigeon HDL, a lipoprotein in which apoA-I is the principal apoprotein, does not compete for binding. The most likely apoprotein responsible for mediating the binding to the β-VLDL receptor is pigeon apoB. ApoB is the major apoprotein of pigeon β-VLDL and LDL, and both of these lipoproteins compete for binding to the β-VLDL receptor on pigeon macrophages. The mammalian β-VLDL receptor will also recognize lipoproteins such as LDL that have apoB as their principal apoprotein, although with a much lower affinity than apoE-containing lipoproteins (22). The affinity for binding of pigeon β-VLDL to the pigeon β-VLDL receptor ranged from 7 to 10 μg/mg of cell protein, values in the same range as described for the binding of rabbit β-VLDL to the mouse peritoneal macrophage (9). As a result, even though pigeon β-VLDL lacks apoE, it binds to the pigeon macrophage with an affinity similar to that for the binding of mammalian β-VLDL to mammalian macrophages. Although pigeon apoB appears the most likely candidate mediating binding to the pigeon β-VLDL receptor, we cannot eliminate a role for one or more of the unidentified small apoproteins of β-VLDL and LDL (20). In addition, it is possible that the apoA-I of pigeon β-VLDL is important for binding, but is in a different conformation on HDL, which prevents HDL from binding to the β-VLDL receptor.

Recent studies have demonstrated that the mammalian β-VLDL receptor is probably an altered form of the LDL receptor (22, 23). The data for the pigeon β-VLDL receptor are consistent with a similar interpretation as seen by the fact that binding and metabolism of pigeon β-VLDL was Ca^{2+}-dependent, pronase-sensitive, down-regulated by cholesterol loading, and blocked by methylation of LDL. These are all classical characteristics of the LDL receptor path-

Downloaded from www.jlr.org by guest, on June 19, 2012

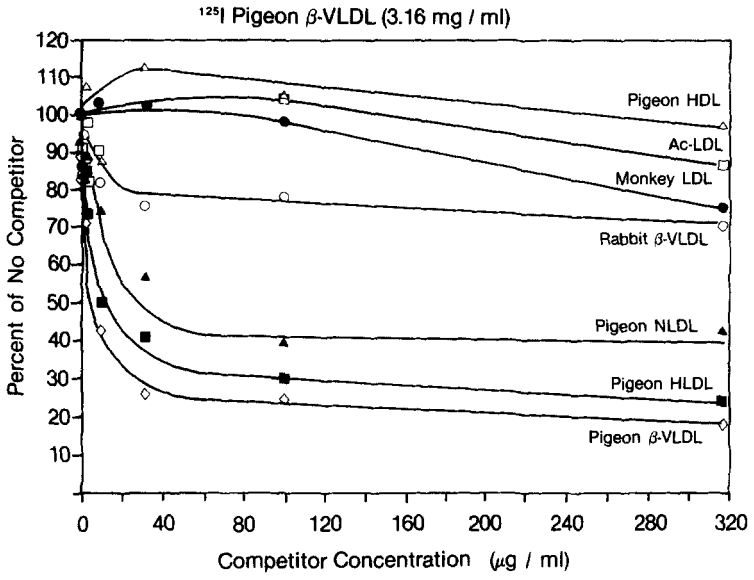

Fig. 7. Competition for binding of ^{125}I-labeled pigeon β-VLDL to WC macrophages at 4°C. β-VLDL was isolated from cholesterol-fed WC pigeons and incubated (3.16 μg/ml) with WC macrophages in the presence of the indicated competitors at a concentration range from 0.316 to 316 μg/ml. Binding in the absence of competitor is shown as 100% and averaged 79.3 ± 6.5 ng bound/mg of cell protein, mean ± SD, n = 6. Duplicate dishes were used at each concentration of the competitors. LDL from normal pigeons is designated as N-LDL. LDL from hypercholesterolemic pigeons is designated as H-LDL.

way (48). An important difference between the pigeon and mammalian system, however, is that pigeon LDL is nearly as effective a competitor as β-VLDL while, in the mammalian system, LDL competes poorly for binding to the β-VLDL receptor (22). This may suggest a difference in the conformation of the β-VLDL receptor or of adjacent proteins on the plasma membrane of pigeon macrophages that allows access of the lipoproteins to the receptor. Equally likely are differences in the conformation of the LDL receptor-binding domain of pigeon LDL (presumably apoB) that make it more accessible to the pigeon macrophage receptor. It should also be emphasized that these studies were done after preincubation of cells with LPDS, a condition that would be expected to maximize β-VLDL/LDL receptor activity. The extent to which this receptor functions in vivo, or whether there are differences between macrophages from the two breeds of pigeon under these conditions, is unknown.

Although the characteristics of binding of β-VLDL are suggestive of binding to an LDL-like receptor, we have shown previously that there is no functional LDL receptor activity on pigeon skin fibroblasts or smooth muscle cells in culture (30, 46). There are several possible explanations for this observation. LDL receptors may be expressed only on some cells of pigeons, or the lack of expression in certain cell types may be an artifact of tissue culture. Alternatively, β-VLDL may bind to a unique receptor (not the LDL

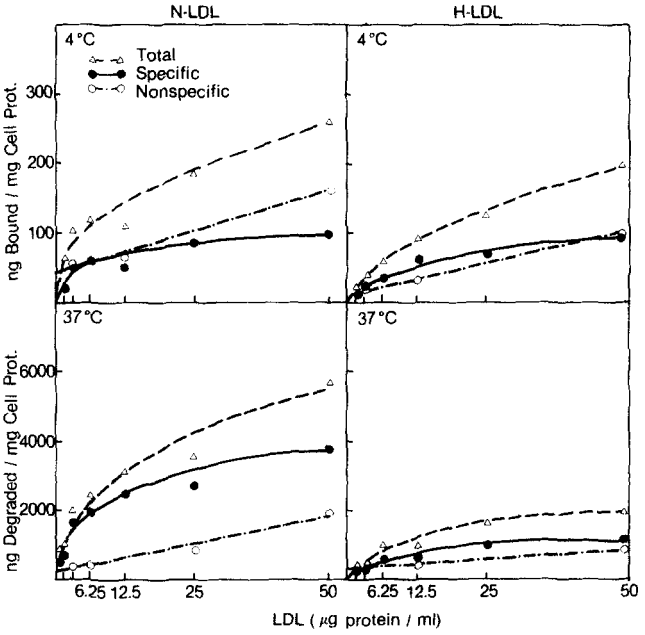

Fig. 8. Concentration dependence of binding and degradation of ^{125}I-labeled LDL from normal and hypercholesterolemic WC pigeons by WC macrophages. Cells were incubated with ^{125}I-labeled LDL in the presence or absence of a 20-fold excess of homologous unlabeled LDL at the indicated concentrations. Binding was determined after 3 hr incubation at 4°C and degradation after 5 hr at 37°C. Normal LDL (N-LDL) was isolated from WC pigeons consuming the cholesterol-free diet and hypercholesterolemic LDL (H-LDL) was isolated from cholesterol-fed WC pigeons. Results are the means of triplicate determinations at each point.

Downloaded from www.jlr.org by guest, on June 19, 2012

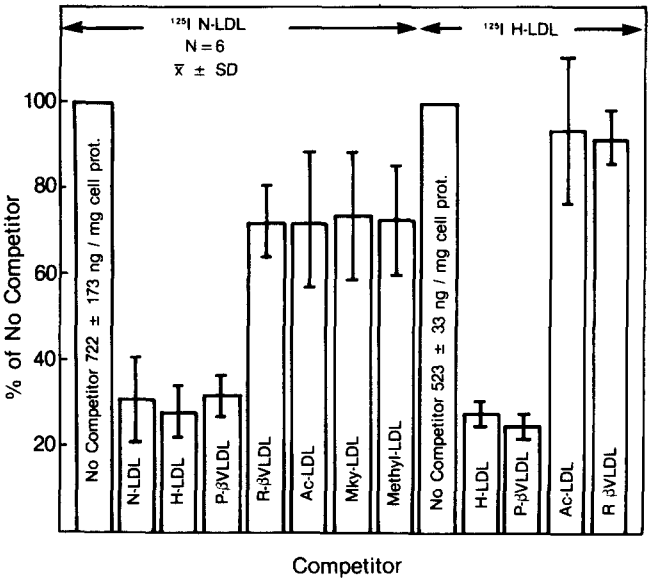

Fig. 9. Competition for metabolism of ^{125}I-labeled pigeon LDL by WC macrophages. LDL were isolated from WC pigeons fed either a cholesterol-free (N-LDL) or cholesterol-containing diet (H-LDL). ^{125}I-Labeled N-LDL or H-LDL (5 μg/ml), in the presence or absence of 100 mg/ml of the indicated competitors, were incubated at 37°C for 5 hr with WC macrophages. Specific LDL metabolism was calculated as the sum of cell-associated and degraded ^{125}I as described in the methods section. The mass (ng) of lipoprotein metabolized per mg of cell protein in the absence of competitor is indicated in the bars labeled "no competitor." This value was set at 100%. The other bars show the mean ± SD (n = 6) for the percent change from "no competitor" experiments for the indicated lipoprotein competitors. The methylated LDL used as competitor in this experiment was prepared from normal pigeon LDL and the monkey LDL (Mky-LDL) was from cholesterol-fed animals, as was the rabbit β-VLDL (R β-VLDL) and pigeon β-VLDL (P β-VLDL).

receptor) on pigeon macrophages that recognizes a ligand common to pigeon β-VLDL and LDL (such as apoB-100). It is clear, however, that whatever the ligand, it is unique to pigeon β-VLDL and LDL, as mammalian LDL and β-VLDL (which contain both apoE and apoB-100) do not compete for binding to the same receptor even though pigeon LDL (46) and β-VLDL (Adelman, S. J., and R. W. St. Clair, unpublished observations) bind to mammalian LDL receptors. LDL from both normal and hypercholesterolemic pigeons compete for binding to the pigeon macrophage β-VLDL receptor, although hypercholesterolemic LDL was somewhat more effective. This may be secondary to the increased size of these LDL resulting from an increase in the number of cholesteryl ester molecules per particle (20). Alternatively, the changes in the size of the LDL could have altered the conformation of the apoB or other proteins, to more closely resemble their conformation on β-VLDL. Since β-VLDL may bind to multiple sites on pigeon macrophages, as evidenced by the curvilinear Scatchard plot, alterations in protein conformation could enhance the ability to bind to one or more of these sites. Furthermore, only hypercholesterolemic LDL promoted cholesteryl ester accumulation. It is not clear whether this was due solely to the higher cholesterol to protein ratio in hypercholesterolemic versus normal LDL (20) or to other properties of these lipoproteins.

These studies demonstrate the presence of at least two lipoprotein receptor pathways (AcLDL and β-VLDL) in pigeon peritoneal macrophages that have the potential to mediate the accumulation of cholesteryl esters and perhaps the development of foam cells in vivo. There were, however, no detectable quantitative or qualitative differences in any of the lipoprotein binding parameters measured between macrophages from WC or SR pigeons. This suggests that differences in susceptibility to atherosclerosis between WC and SR pigeons cannot be explained by a genetically mediated difference in the expression of these receptors. Nevertheless, since pigeon peritoneal macrophages and monocyte macrophages (49) express both AcLDL and β-VLDL recep-

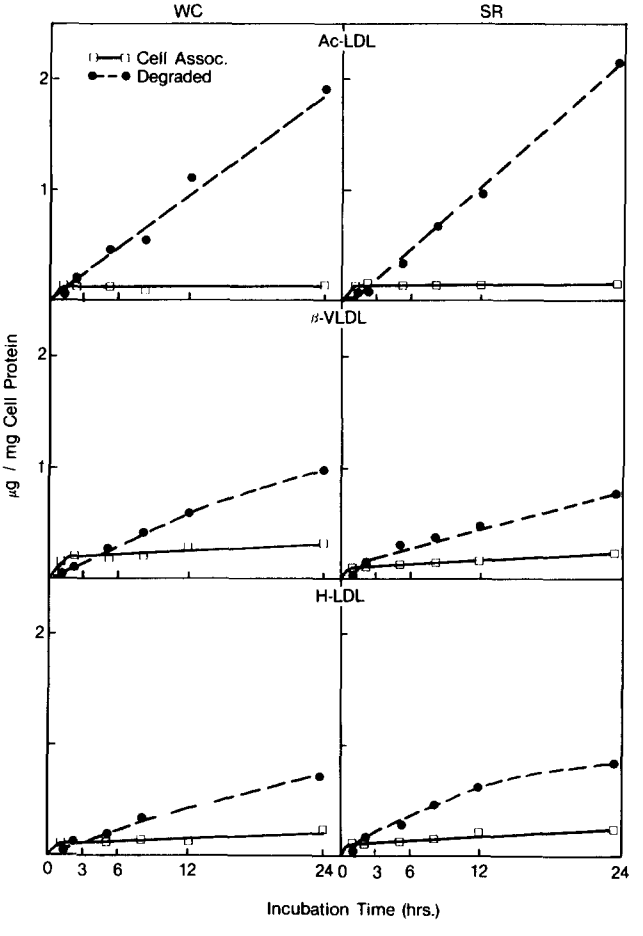

Fig. 10. Time course of lipoprotein metabolism by WC and SR macrophages. Cells were incubated with 5 μg/ml of ^{125}I-labeled AcLDL, β-VLDL, or H-LDL (LDL from hypercholesterolemic animals) from WC pigeons. Incubations were carried out at 37°C for the indicated times. Results are the means of triplicate determinations at each time point.

Downloaded from www.jlr.org by guest, on June 19, 2012

TABLE 2. Effect of lipoproteins on cholesterol accumulation and esterification in thioglycolate-elicited peritoneal macrophages from WC and SR pigeons

	White Carneau			Show Racer		
		Chol. Accum.			Chol. Accum.	
	CE Synthesis	FC	EC	CE Synthesis	FC	EC
	nmol/mg prot.	*μg/mg prot.*		*nmol/mg prot.*	*μg/mg prot.*	
Expt. 1 (n = 2)						
LPDS	10	36	13			
Mky AcLDL						
100 μg/ml	76	37	95			
200 μg/ml	86	34	112			
WC AcLDL						
56 μg/ml	50	32	71			
113 μg/ml	64	34	145			
Expt. 2 (n = 3)						
LPDS	1	15	1	1	15	7
FBS	1	14	2	1	15	4
AcLDL						
200 μg/ml	3	21	41	3	20	29
WC β-VLDL						
50 μg/ml	18	22	104	12	23	92
Expt. 3 (n = 3)						
LPDS	4	16	1	8	16	8
WC N-LDL						
200 μg/ml	5	14	4	11	13	12
WC H-LDL						
200 μg/ml	31	20	91	40	21	111

WC pigeon macrophages were incubated for 24 hr in medium containing LPDS (2.5 μg/ml). Cells were washed two times with PBS then incubated with medium containing the lipoproteins at the protein concentrations indicated. For measurement of cholesteryl ester synthesis (CE synthesis), cells were incubated for 24 hr in the presence of the lipoproteins plus [1-^{14}C]oleate. For measurement of cell cholesterol content, cells were incubated with the indicated lipoproteins for 48 hr and then assayed for free (FC) and esterified (EC) cholesterol content by GLC. In experiment 1, the monkey and pigeon AcLDL had a cholesterol to protein ratio of 2.24 and 4.0, respectively. In experiment 2, the monkey AcLDL had a cholesterol to protein ratio of 2.3, while the ratio was 9.32 for WC pigeon β-VLDL. In experiment 3, normal WC pigeon LDL (N-LDL) and LDL from hypercholesterolemic WC pigeons (H-LDL) had cholesterol to protein ratios of 1.5 and 4.1, respectively. Results are the mean of the indicated number (n) of replicate dishes.

tors, the mechanisms exist for the development of foam cells within the arterial wall as a result of the interaction of plasma lipoproteins with macrophages. Whether the difference in atherosclerosis susceptibility between WC and SR pigeons is related to differences in the entry of monocytes into the arterial wall or to localized factors affecting either the lipoproteins that normally filter through the arterial wall or the macrophages themselves remains to be elucidated.

We acknowledge the excellent technical assistance of Ms. Molly Leight and Mrs. Patricia Hester, and the assistance of Mrs. Barbara Lindsay and Patricia Brooks in the preparation of this manuscript. This work was supported by SCOR Grant HL-14164 from the National Heart, Lung, and Blood Institute. Dr. Adelman was supported by National Research Service Award Institutional Grant HL-07115.

Manuscript received 11 September 1987 and in revised form 16 November 1987.

REFERENCES

1. Lang, P. D., and W. Insull, Jr. 1970. Lipid droplets in atherosclerotic fatty streaks of human aorta. *J. Clin. Invest.* **49:** 1479–1488.
2. Smith, E. B., P. H. Evans, and M. D. Downsham. 1967. Lipid in the aortic intima: the correlation of morphologic and chemical characteristics. *J. Atheroscler. Res.* **7:** 171–186.
3. St. Clair, R. W. 1976. Metabolism of the arterial wall and atherosclerosis. *Atheroscler. Rev.* **1:** 61–117.
4. Buja, L. M., T. Kita, J. L. Goldstein, Y. Watanabe, and M. S. Brown. 1983. Cellular pathology of progressive atherosclerosis in the WHHL rabbit. An animal model of familial hypercholesterolemia. *Arteriosclerosis.* **3:** 87–101.
5. Tsukada, T., M. Rosenfeld, R. Ross, and A. M. Gown. 1986. Immunocytochemical analysis of cellular components in atherosclerotic lesions: use of monoclonal antibodies with the Watanabe and fat-fed rabbit. *Arteriosclerosis.* **6:** 601–613.
6. Rosenfeld, M. E., T. Tsukada, A. Chait, E. L. Beirman, A. M. Gown, and R. Ross. 1987. Fatty streak expansion and maturation in Watanabe heritable hyperlipemic and com-

Downloaded from www.jlr.org by guest, on June 19, 2012

parably hypercholesterolemic fat-fed rabbits. *Arteriosclerosis.* **7:** 24–34.
7. Faggiotto, A., R. Ross, and L. Harker. 1984. Studies of hypercholesterolemia in the nonhuman primate. I. Changes that lead to fatty streak formation. *Arteriosclerosis.* **4:** 323–340.
8. Faggiotto, A., and R. Ross. 1984. Studies of hypercholesterolemia in the nonhuman primate. II. Fatty streak conversion to fibrous plaque. *Arteriosclerosis.* **4:** 341–356.
9. Brown, M. S., and J. L. Goldstein. 1983. Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. *Annu. Rev. Biochem.* **52:** 223–261.
10. Mahley, R. W., and T. L. Innerarity. 1983. Lipoprotein receptors and cholesterol homeostasis. *Biochim. Biophys. Acta.* **737:** 197–222.
11. Goldstein, J. L., Y. K. Ho, S. K. Basu, and M. S. Brown. 1974. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc. Natl. Acad. Sci. USA.* **76:** 333–377.
12. Mahley, R. W., T. L. Innerarity, K. H. Weisgraber, and S. Y. Oh. 1979. Altered metabolism (in vivo and in vitro) of plasma lipoproteins after selective chemical modification of lysine residues of the apoproteins. *J. Clin. Invest.* **64:** 743–750.
13. Fogelman, A. M., I. Shechter, J. Seager, M. Hokom, J. S. Child, and P. A. Edwards. 1980. Malondialdehyde alteration of low density lipoproteins leads to cholesteryl ester accumulation in human monocyte-macrophages. *Proc. Natl. Acad. Sci. USA.* **77:** 2214–2218.
14. Henriksen, T., E. M. Mahoney, and D. Steinberg. 1981. Enhanced macrophage degradation of low density lipoprotein previously incubated with cultured endothelial cells: recognition by receptors for acetylated low density lipoproteins. *Proc. Natl. Acad. Sci. USA.* **78:** 6499–6503.
15. Heinecke, J. W., L. Baker, H. Rosen, and A. Chait. 1986. Superoxide-mediated modification of low density lipoprotein by arterial smooth muscle cells. *J. Clin. Invest.* **77:** 757–761.
16. Morton, R. E., G. A. West, and H. F. Hoff. 1986. A low density lipoprotein-sized particle isolated from human atherosclerotic lesions is internalized by macrophages via a non-scavenger-receptor mechanism. *J. Lipid Res.* **27:** 1124–1134.
17. Brown, M. S., J. L. Goldstein, and D. S. Fredrickson. 1983. Familial type 3 hyperlipoproteinemia. *In* The Metabolic Basis of Inherited Disease. J. B. Stanbury, J. B. Wyngaarden, D. S. Fredrickson, J. L. Goldstein, and M. S. Brown, editors. McGraw-Hill, New York. 655–671.
18. Mahley, R. W., K. H. Weisgraber, and T. L. Innerarity. 1974. Canine lipoproteins and atherosclerosis. II. Characterization of the lipoproteins associated with atherogenic and nonatherogenic hyperlipidemia. *Circ. Res.* **35:** 722–733.
19. Mahley, R. W., T. L. Innerarity, M. S. Brown, Y.K. Ho, and J. L. Goldstein. 1980. Cholesteryl ester synthesis in macrophages: stimulation by β-very low density lipoproteins from cholesterol-fed animals of several species. *J. Lipid Res.* **21:** 970–980.
20. Barakat, H. A., and R. W. St. Clair. 1985. Characterization of plasma lipoproteins of grain- and cholesterol-fed White Carneau and Show Racer pigeons. *J. Lipid Res.* **26:** 1252–1268.
21. Innerarity, T. L., K. S. Arnold, K. H. Weisgraber, and R. W. Mahley. 1986. Apolipoprotein E is the determinant that mediates the receptor uptake of β-very low density lipoproteins by mouse macrophages. *Arteriosclerosis.* **6:** 114–122.
22. Koo, C., M. E. Wernette-Hammond, and T. L. Innerarity. 1986. Uptake of canine β-very low density lipoproteins by mouse peritoneal macrophages is mediated by a low density lipoprotein receptor. *J. Biol. Chem.* **261:** 11194–11201.
23. Ellsworth, J. L., F. B. Kraemer, and A. D. Cooper. 1987. Transport of β-very low density lipoproteins and chylomicron remnants by macrophages is mediated by the low density lipoprotein receptor pathway. *J. Biol. Chem.* **262:** 2316–2325.
24. St. Clair, R. W. 1983. Metabolic changes in the arterial wall associated with atherosclerosis in the pigeon. *Fed. Proc.* **42:** 2480–2485.
25. Wagner, W. D., T. B. Clarkson, M. A. Feldner, and R. W. Prichard. 1973. The development of pigeon strains with selected atherosclerosis characteristics. *Exp. Mol. Pathol.* **19:** 304–319.
26. Prichard, R. W., T. B. Clarkson, H. O. Goodman, and H. B. Lofland. 1964. Aortic atherosclerosis in pigeons and its complications. *Arch. Pathol.* **77:** 244–257.
27. Taylor, R. G., and J. C. Lewis. 1986. Endothelial cell proliferation and monocyte adhesion to atherosclerotic lesions of White Carneau pigeons. *Am. J. Pathol.* **125:** 152–160.
28. Jerome, W. G., and J. C. Lewis. 1985. Early atherosclerosis in White Carneau pigeons. II. Ultrastructural and cytochemical observations. *Am. J. Pathol.* **119:** 210–222.
29. St. Clair, R. W., R. K. Randolph, M. P. Jokinen, T. B. Clarkson, and H. A. Barakat. 1986. Relationship of plasma lipoproteins and the monocyte–macrophage system to atherosclerosis severity in cholesterol-fed pigeons. *Arteriosclerosis.* **6:** 614–626.
30. Randolph, R. K., and R. W. St. Clair. 1984. Pigeon aortic smooth muscle cells lack a functional low density lipoprotein receptor pathway. *J. Lipid Res.* **25:** 888–902.
31. St. Clair, R. W., J. J. Mitschelen, and M. Leight. 1980. Metabolism by cells in culture of low-density lipoproteins of abnormal composition from non-human primates with diet-induced hypercholesterolemia. *Biochim. Biophys. Acta.* **618:** 63–79.
32. Rudel, L. L., J. A. Lee, M. D. Morris, and J. M. Felts. 1974. Characterization of plasma lipoproteins separated and purified by agarose-column chromatography. *Biochem. J.* **139:** 89–95.
33. Basu, S. K., J. L. Goldstein, R. G. W. Anderson, and M. S. Brown. 1976. Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. *Proc. Natl. Acad. Sci. USA.* **73:** 3178–3182.
34. Weisgraber, K. H., T. L. Innerarity, and R. W. Mahley. 1978. Role of the lysine residues of plasma lipoproteins in high affinity binding to cell surface receptors on human fibroblasts. *J. Biol. Chem.* **253:** 9053–9062.
35. McFarlane, A. S. 1964. Metabolism of plasma proteins. *In* Mammalian Protein Metabolism. H. N. Munro, and J. B. Allison, editors. Vol. 1. Academic Press, New York. 297–341.
36. Goldstein, J. L., and M. S. Brown. 1974. Binding and degradation of low density lipoproteins by cultured human fibroblasts. Comparison of cells from a normal subject and from a patient with homozygous familial hypercholesterolemia. *J. Biol. Chem.* **249:** 5153–5162.
37. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
38. Smith, B. P., R. W. St. Clair, and J. C. Lewis. 1979. Cholesterol esterification and cholesteryl ester accumulation in cultured pigeon and monkey arterial smooth muscle cells. *Exp. Mol. Pathol.* **30:** 190–208.
39. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.

ASBMB JOURNAL OF LIPID RESEARCH
Downloaded from www.jlr.org by guest, on June 19, 2012

40. Ishikawa, T. T., J. MacGee, J. A. Morrison, and C. J. Glueck. 1974. Quantitative analysis of cholesterol in 5 to 20 μl of plasma. *J. Lipid Res.* **15:** 286–291.
41. St. Clair, R. W., and M. A. Leight. 1978. Differential effects of isolated lipoproteins from normal and hypercholesterolemic rhesus monkeys in culture. *Biochim. Biophys. Acta.* **530:** 279–291.
42. Vijayagopal, P., S. R. Srinivasan, K. M. Jones, B. Radhakrishnamurthy, and G. S. Berenson. 1985. Complexes of low-density lipoproteins and arterial proteoglycan aggregates promote cholesteryl ester accumulation in mouse macrophages. *Biochim. Biophys. Acta.* **837:** 251–261.
43. Clevidence, B. A., R. E. Morton, G. West, D. M. Dusek, and H. F. Hoff. 1984. Cholesterol esterification in macrophages: stimulation by lipoproteins containing apoB isolated from human aortas. *Arteriosclerosis.* **4:** 196–207.
44. Kita, T., M. S. Brown, Y. Watanabe, and J. L. Goldstein. 1981. Deficiency of low density lipoprotein receptors in liver and adrenal gland of the WHHL rabbit, an animal model of familial hypercholesterolemia. *Proc. Natl. Acad. Sci. USA.* **78:** 2268–2272.
45. Marzetta, C. A., and L. L. Rudel. 1986. A species comparison of low density lipoprotein heterogeneity in nonhuman primates fed atherogenic diets. *J. Lipid Res.* **27:** 753–762.
46. St. Clair, R. W., M. A. Leight, and H. A. Barakat. 1986. Metabolism of low density lipoproteins by pigeon fibroblasts and aortic smooth muscle cells. Comparison of cells from atherosclerosis-susceptible and atherosclerosis-resistant pigeons. *Arteriosclerosis.* **6:** 170–177.
47. Rajavashisth, T. B., P. A. Dawson, D. L. Williams, J. E. Shackelford, H. Lebherz, and A. J. Lusis. 1987. Structure, evolution, and regulation of chicken apolipoprotein A-I. *J. Biol. Chem.* **262:** 7058–7065.
48. Goldstein, J. L. and M. S. Brown. 1977. The low density lipoprotein pathway and its relation to atherosclerosis. *Annu. Rev. Biochem.* **46:** 897–930.
49. Henson, D. A., J. C. Lewis, and R. W. St. Clair. 1986. Lipoprotein metabolism by pigeon monocyte-derived macrophages (PMDM) in culture. *Fed. Proc.* **45:** 692(abstract).

Downloaded from www.jlr.org by guest, on June 19, 2012