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# The role of lipoproteins and receptor-mediated endocytosis in the transport of bacterial lipopolysaccharide

(endotoxin/atherosclerosis)

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ABSTRACT **The addition of bacterial lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 to human monocyte-macrophages cultured in serum results in suppression of scavenger receptor activity. The present studies were performed to examine if the effect on scavenger receptor activity was mediated by LPS alone or by LPS in association with lipoproteins. Radioiodinated LPS ($^{125}$I-LPS) was added to human plasma *in vitro* and to normal and hyperlipidemic rabbit plasma *in vitro* and *in vivo* to determine the distribution of $^{125}$I-LPS among the lipoprotein classes. It was found that all lipoprotein classes bound LPS in direct proportion to their plasma cholesterol concentration. LPS alone was compared to LPS bound to low density lipoprotein (LDL), high density lipoprotein, or reductively-methylated LDL for their abilities to suppress scavenger receptor activity in monocyte-macrophages in lipoprotein-free serum. Only LPS bound to LDL (LPS–LDL) demonstrated an effect similar to that observed when LPS was added to cells in serum. Either unlabeled LDL or unlabeled LPS–LDL complexes competed with the uptake of $^{125}$I-LPS–LDL complexes, which appeared to proceed by receptor-mediated endocytosis. In contrast to the uptake of $^{125}$I-LDL, the uptake of $^{125}$I-LPS–LDL by cultured monocyte-macrophages was not followed by its hydrolysis and the release of its radioactive degradation products into the medium. The association of LPS with lipoproteins was very stable and appeared to be mediated by a lipid–lipid interaction. We hypothesize that LPS bound to lipoproteins may be transported into the artery wall and may initiate the atherosclerotic reaction.**

It was initially shown by Ulevitch and Johnston that incubation of bacterial lipopolysaccharide (LPS) with serum could modify its endotoxic properties and produce a shift in the hydrated density of the LPS (1). Subsequently, it was demonstrated that lipoproteins in the serum were able to bind LPS (2–4). These studies of lipoprotein–LPS interactions have focused on the role of high density lipoproteins (HDL) and have utilized rats or rabbits as experimental models, species in which HDL carries most of the plasma cholesterol. Studies from this laboratory (5) found that, in addition to HDL, low density lipoproteins (LDL) markedly reduced the cytotoxic effects of LPS added to bovine aortic endothelial cell cultures compared to cells that did not have lipoproteins present in the medium. These results have recently been confirmed by others (6).

We have shown (7) that LPS added to human monocyte-macrophages cultured in serum resulted in suppression of scavenger receptor activity. It was not determined, however, if this suppression was mediated by LPS alone or by LPS associated with serum lipoproteins. In the present investigation, we studied the interaction between LPS and plasma lipoproteins. We demonstrate that (*i*) all major lipoprotein classes bind LPS in the blood in direct proportion to their plasma cholesterol concentration; (*ii*) the association of LPS with lipoproteins is remarkably stable; (*iii*) a lipid–lipid interaction appears to account for the association of LPS with lipoproteins; (*iv*) the uptake of LPS–LDL complexes proceeds by apolipoprotein B/E receptor-mediated endocytosis and results in suppression of scavenger (malondialdehyde altered-LDL) receptor activity.

## MATERIALS AND METHODS

**Materials.** The LPS (from *Escherichia coli* strain 0111:B4) and chloramine-T were purchased from Sigma. Sepharose CL-2B was purchased from Pharmacia. *p*-Hydroxymethylbenzimidate (Wood's reagent) was purchased from Pierce Biochemicals. Polyvalent *E. coli* antiserum was purchased from Baltimore Biological Laboratories, Microbiology Systems (Cockeysville, MD). All other supplies and reagents were obtained from sources as reported (7).

**Human Subjects.** Normal fasted subjects were recruited from the staff and student body at UCLA. Informed consent was obtained in writing from each person.

**Rabbits.** Normal New Zealand White (NZW) male rabbits, NZW rabbits fed a 2% cholesterol diet, NZW rabbits infused with 160 mg of rabbit LDL protein per kg of body weight, or Watanabe heritable hyperlipidemic (WHHL) rabbits were used in these studies (8). All rabbits were fasted for 24 hr before experiments were begun.

**Separation and Culture of Cells.** Monocytes were prepared by counterflow centrifugation, classified, and cultured, and their viability was determined as described (9).

**Lipoprotein Isolation and Preparation.** Very low density lipoproteins (VLDL) (density < 1.006 g/ml), LDL (density = 1.019–1.063 g/ml), and HDL (density = 1.063–1.21 g/ml) were isolated by the method of Havel *et al.* (10) from either rabbit or human plasma after a fast. Lipoproteins were radiolabeled as described (11, 12). Malondialdehyde-treated LDL and reductively methylated LDL (ReMe-LDL) were prepared as described (13).

**Column Chromatography.** Sepharose CL-2B was used to separate LDL, LPS, and LPS–LDL complexes. Chromatography was carried out with 1-ml sample aliquots containing 1 mg of LPS in lipoprotein-deficient serum or 1 mg of LDL or LPS–LDL protein applied to a 0.9 × 60 cm column. The samples were eluted in 0.15 M NaCl/10 mM phosphate/0.01% EDTA, pH 7.4, at 23°C and at a flow rate of 20 ml/hr. The void volume was 36.3 ml, and fractions of 0.5 ml were collected.

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Abbreviations: LPS, lipopolysaccharide; HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoproteins; NZW, New Zealand White; WHHL, Watanabe heritable hyperlipidemic; ReMe-LDL, reductively methylated low density lipoprotein.

**LPS Preparation.** The LPS used in these studies was modified by *p*-hydroxymethylbenzimidate by the method of Ulevitch (14). The derivatized LPS was radiolabeled as described (14). Ulevitch has previously demonstrated that these procedures do not alter the biologic properties of LPS (1). Additionally, we determined that injection of $^{125}$I-labeled LPS ($^{125}$I-LPS) into rabbits produced pathologic evidence of classic endotoxic shock identical to that obtained with nonradioactive LPS.

**Preparation of LPS–Lipoprotein Complexes for Cell Culture Studies.** In experiments not shown, 5 mg of lipoprotein-protein was incubated with 0.1–5 mg of $^{125}$I-LPS in 10 ml of lipoprotein-deficient serum using a rotating shaker (Labquake, Labindustries, Berkeley, CA) for 6 hr at 37°C. At the end of this time, the serum mixture was adjusted to the particular hydrated density for the lipoprotein class desired, and ultracentrifugation was carried out. It was consistently found that a maximum of 90–100 ng of $^{125}$I-LPS was associated per μg of lipoprotein-protein as the amount of added $^{125}$I-LPS approached 5 mg. Therefore, to prepare $^{125}$I-LPS-lipoproteins complexes, 5 mg of $^{125}$I-LPS was incubated with 5 mg of lipoprotein-protein in 10 ml of lipoprotein-deficient serum to provide optimal conditions for association.

**Assays.** The protein content of cells and lipoproteins was determined by the method of Lowry *et al.* (15).

Prior to initiating studies, the cells were washed three times with 1-ml volumes of Dulbecco's modified Eagle's medium containing 10 mM Hepes (pH 7.4) (medium C). Radioactive lipoproteins were added to the cells in medium C supplemented with 25 mM $NaHCO_3$ and glucose at 2 mg/ml (medium D).

The proteolytic degradation of $^{125}$I-LDL and $^{125}$I-labeled malondialdehyde-treated LDL was measured as acid-soluble radioactivity as described (9, 16). For studies measuring cell-associated radioactivity, the cells were sequentially washed four times with 1 ml of phosphate-buffered saline Gibco Laboratories (Chagrin Falls, OH) containing 0.2% bovine serum albumin and once with 1 ml of phosphate-buffered saline; the first wash was allowed to sit 10 min before proceeding with the remaining washes in rapid succession (9). Each well then received 0.75 ml of 0.1 M sodium hydroxide; after 30 min at 20°C, the cellular extract was quantitatively transferred to vials for determination of radioactivity. Wells without cells were treated identically to serve as controls.

## RESULTS

**Distribution of $^{125}$I-LPS among the Lipoprotein Fractions.** Incubation of plasma from normal humans with $^{125}$I-LPS resulted in most of the $^{125}$I-LPS partitioned primarily between LDL and HDL (Table 1). When plasma from NZW rabbits was incubated with $^{125}$I-LPS, the lipoprotein fraction associated with the most $^{125}$I-LPS was the HDL. Since the majority of plasma cholesterol is contained in the HDL fraction in NZW rabbits, we determined if incubation of $^{125}$I-LPS with plasma from rabbits that have elevated levels of other lipoproteins would result in a different distribution. In contrast to NZW rabbits, most of the $^{125}$I-LPS in plasma from WHHL and cholesterol-fed rabbits was associated with VLDL and LDL, the major cholesterol-containing lipoproteins of these rabbits.

Similar results were found when $^{125}$I-LPS was injected into rabbits and the plasma was removed and fractionated (Table 2). In NZW rabbits the lipoprotein fraction that contained the most $^{125}$I-LPS was HDL. When a NZW rabbit was infused with LDL to increase this lipoprotein fraction to levels comparable to that of a WHHL rabbit (600 mg of cholesterol/dl), most of the injected $^{125}$I-LPS was found in the isolated LDL fraction. $^{125}$I-LPS injected into WHHL and cholesterol-fed rabbits was distributed mainly between the VLDL and LDL fractions, as was observed in the *in vitro* experiments (Table 1). We conclude that the distribution of LPS in the blood is determined by the concentration of the cholesterol-rich lipoprotein(s) present in the plasma.

Table 1. *In vitro* distribution of $^{125}$I-LPS among the lipoprotein classes of human and rabbit plasma

| | $^{125}$I-LPS distribution,* % of total | | | |
|---|---|---|---|---|
| | | Rabbit | | |
| Lipoprotein class | Human | NZW | WHHL | CF |
| VLDL | 12 (9) | 9 (30) | 27 (37) | 35 (43) |
| LDL | 34 (36) | 14 (25) | 49 (60) | 35 (48) |
| HDL | 22 (55) | 36 (45) | 7 (3) | 9 (9) |
| $d > 1.21$ g/ml | 32 | 41 | 17 | 21 |

Plasma samples (10 ml) from fasted normal humans or from NZW, WHHL, or cholesterol-fed (CF) rabbits were incubated with 50 μg of $^{125}$I-LPS (210 cpm/ng) for 6 hr at 37°C. Samples were then fractionated by ultracentrifugation into the designated classes. *d*, Density.
*Values are the means of two separate incubations and represent the percentage of total radioactivity in the original plasma sample recovered in each class. Values in parentheses indicate the percentage of total lipoprotein-protein in each density class.

**Interaction of LPS–Lipoprotein Complexes with Monocyte-Macrophages.** We had shown in an earlier study (7) that incubation of human monocyte-macrophages with 10–100 ng of LPS per ml of serum medium inhibited the expression of scavenger receptor activity. It was not known, however, if LPS exerted its effects by direct uptake by the cell or if the effect was mediated by uptake of LPS complexed with lipoproteins. To test this, $^{125}$I-LPS was incubated with lipoproteins to determine by radioactivity the content of LPS associated with the lipoproteins in the complexes. Fig. 1 demonstrates that addition of increasing amounts of $^{125}$I-LPS–LDL that contained 10–100 ng of LPS per ml to cells in lipoprotein-deficient serum produced a progressive suppression of scavenger receptor activity as assayed by degradation of endocytosed malondialdehyde-treated LDL. This effect was not seen when the same amount of LPS was added in a complex with either ReMe-LDL or HDL. Moreover, there was only a 38% suppression when LPS was added directly to lipoprotein-deficient serum. The protein content of the cells and their viability were not different whether $^{125}$I-LPS–LDL, $^{125}$I-LPS–ReMe-LDL, $^{125}$I-LPS–HDL, or LPS alone was added. These data indicate that the $^{125}$I-LPS–LDL produced a biologic response similar to that previously shown for unlabeled LPS added to serum (7).

Fig. 2 shows that the uptake of $^{125}$I-LPS–LDL by monocyte-macrophages could be suppressed by unlabeled

Table 2. *In vivo* distribution of $^{125}$I-LPS among the lipoprotein classes of rabbits

| | $^{125}$I-LPS distribution in rabbits,* % of total | | | |
|---|---|---|---|---|
| Lipoprotein class | NZW | LDL-infused NZW | CF | WHHL |
| VLDL | 23 (29) | 24 (23) | 56 (42) | 35 (38) |
| LDL | 21 (24) | 42 (44) | 24 (49) | 46 (59) |
| HDL | 34 (47) | 24 (33) | 10 (9) | 7 (3) |
| $d > 1.21$ | 22 | 10 | 10 | 12 |

$^{125}$I-LPS (1 mg) was injected into each rabbit via a marginal ear vein, and blood was drawn 3 hr later. Plasma samples were then fractionated by ultracentrifugation into the designated classes. CF, cholesterol-fed rabbits; *d*, density.
*Values represent the percentage of total radioactivity in the original plasma sample recovered in each class. Values in parentheses indicate the percentage of total lipoprotein-protein in each density class.

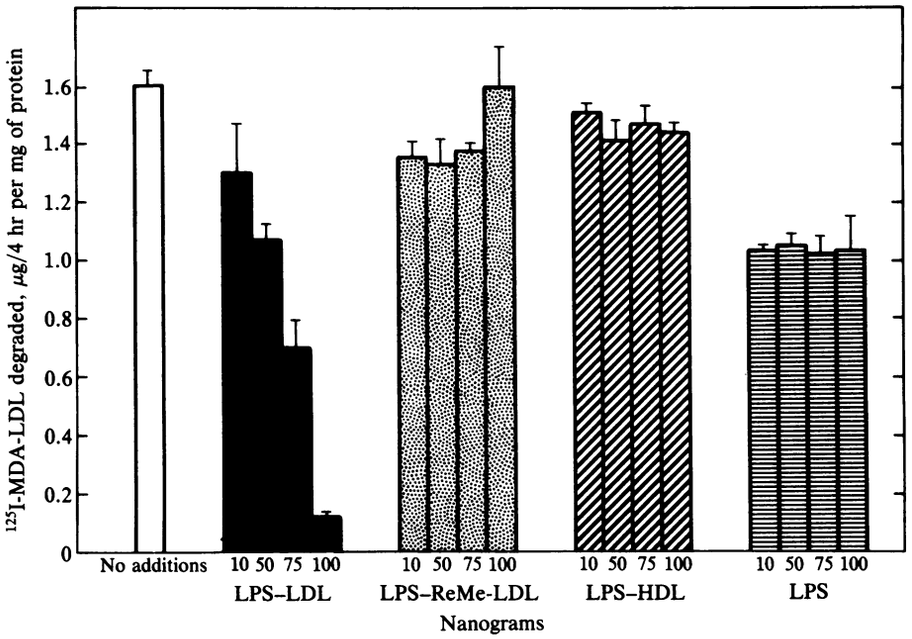


FIG. 1. The effects of various LPS–lipoprotein complexes on the expression of scavenger receptor activity in monocyte-macrophages. Normal human monocytes were cultured in 30% autologous serum in 0.5 ml of medium B. After 2 days the medium was changed, and 10, 50, 75, or 100 ng of $^{125}$I-LPS per ml (▤), or the same amounts complexed with either LDL (■), ReMe-LDL (▥), or HDL (▨) were added in lipoprotein-deficient serum. Three days later, the medium was removed and the cells were washed three times with 1 ml of medium C. Then 0.5 ml of medium D containing 5 μg of $^{125}$I-labeled malondialdehyde-treated LDL ($^{125}$I-MDA-LDL) per ml (220 cpm/ng of protein) was added. After 4 hr of incubation at 37°C, the content of $^{125}$I-labeled acid-soluble material in the medium was determined. Parallel incubations with unlabeled MDA-LDL demonstrated no significant $^{125}$I-labeled acid-soluble material, indicating that the $^{125}$I-labeled acid-soluble material resulted from the lysosomal degradation of $^{125}$I-MDA-LDL and not from the preincubation with $^{125}$I-LPS–LDL. Values are expressed as the mean ± 1 SD of quadruplicate wells.

LDL as well as by unlabeled LPS–LDL but not with unlabeled malondialdehyde-treated LDL, a ligand recognized by the scavenger receptor. Further evidence for a possible role of the LDL receptor in the uptake of $^{125}$I-LPS–LDL is presented in Fig. 3. The association of $^{125}$I-LPS–LDL with monocytes–macrophages as a function of protein concentration resembles the receptor-mediated uptake of $^{125}$I-LDL by these cells (13).

$^{125}$I-LDL is taken up by the LDL receptor pathway and mono[$^{125}$I]iodotyrosine is released from the cells into the medium after protein degradation (16). In contrast, radioactivity from $^{125}$I-LPS–LDL remained associated with the cells and was not released into the medium (Table 3). Radioactiv-

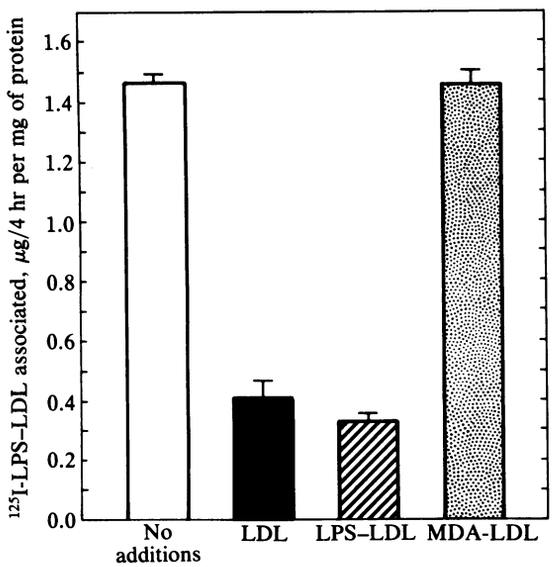


FIG. 2. The ability of LDL, LPS–LDL, or malondialdehyde-treated LDL (MDA-LDL) to inhibit the cell-association of $^{125}$I-LPS–LDL. Normal human monocytes were cultured in 30% autologous serum in 0.5 ml of medium B for 7 days and were prepared for studies as described. Each well received 0.5 ml of medium D containing protein of $^{125}$I-LPS–LDL (200 cpm/ng) at 20 μg/ml alone (□) or together with a 25-fold excess of either unlabeled LDL (■), LPS–LDL (▨), or MDA-LDL (▥). After incubation for 4 hr at 37°C, the medium was removed, the cells were washed, and the $^{125}$I-LPS–LDL associated with the cells was determined as described. The values represent the mean ± 1 SD of quadruplicate wells.

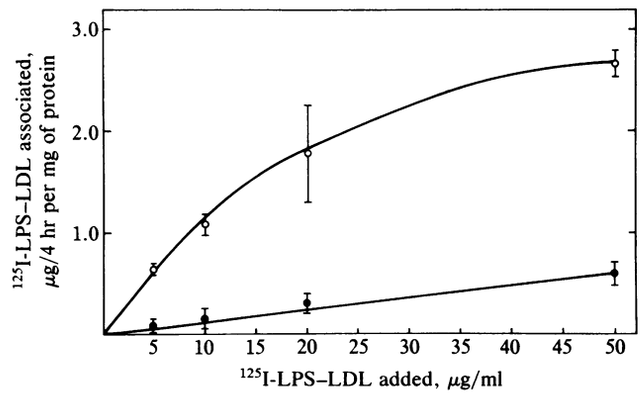


FIG. 3. Cell association of $^{125}$I-LPS–LDL as a function of concentration. Normal human monocytes were cultured in 30% autologous serum in 0.5 ml of medium B for 7 days and were prepared for studies as described. Each well received 0.5 ml of medium D containing the protein concentration of $^{125}$I-LPS–LDL (200 cpm/ng) shown on the abscissa with (●) or without (○) a 25-fold excess of nonradioactive LDL. After incubation for 4 hr at 37°C, the medium was removed, the cells were washed, and the $^{125}$I-LPS–LDL associated with the cells was determined as described. The values represent the mean ± 1 SD of quadruplicate wells.

ity from $^{125}$I-LDL progressively increased in the medium and decreased in the cells within the same period of time.

**Evidence for LPS–Lipoprotein Association.** The molecular mass of *E. coli* 0111:B4 LPS has been reported to be 1 MDa (17), whereas LDL has been reported as having a molecular mass of 2–3 MDa (18). To demonstrate that the $^{125}$I being measured and traced in these studies was indeed linked to LPS–lipoprotein complexes, the radioactive compounds were subjected to gel filtration (Fig. 4). The fraction from WHHL plasma with a density of 1.019–1.063 g/ml (the LDL fraction) contained a broad spectrum of particles. However, after incubation with LDL in the lipoprotein-deficient fraction of serum, there was a shift in the distribution of the radioactivity from smaller ($^{125}$I-LPS) to larger particles (Fig. 4), consistent with the formation of $^{125}$I-LPS–LDL complexes. As a further means of identification, we used the ring (interfacial) test (19) to immunologically determine the presence of LPS in the $^{125}$I-LPS–LDL complex. A precipitin ring formed at the interface between the layer containing the $^{125}$I-LPS–LDL complex and an antiserum to *E. coli* 0111:B4, confirming the presence of LPS in the radioiodinated complex. No precipitin ring was seen when LDL alone was tested. The data from Fig. 4 together with the shift in density of $^{125}$I-LPS from >1.21 g/ml (see below) to 1.019–1.063 g/ml upon incubation with LDL in lipoprotein-deficient serum and the immunologic proof that this fraction contained LPS provide strong evidence that $^{125}$I-LPS associates with LDL to form $^{125}$I-LPS–LDL complexes.

**Characteristics of the LPS–Lipoprotein Interaction.** Reductive methylation of LDL did not affect the association of $^{125}$I-LPS with the lipoprotein. Upon ultracentrifugal fractionation, 75% of the radioactivity was isolated in the fraction with a density of 1.019–1.063 g/ml whether ReMe-LDL or native LDL was incubated with $^{125}$I-LPS in lipoprotein-deficient serum. When, however, $^{125}$I-LPS, with a reported density of 1.44 g/ml (2), was incubated without lipoproteins present in the mixture, 99.6% of the radioactivity was found in the fraction with a density of >1.21 g/ml upon reisolation. More than 95% of the radioactivity from $^{125}$I-LPS–LDL was extracted into chloroform/methanol as opposed to that from $^{125}$I-LDL, of which <2% was extracted into chloroform/methanol. These results suggest that a lipid–lipid interaction accounts for the association of LPS with lipoproteins, as has been suggested for the interaction of LPS with cell membranes (20).

Table 3. Cell-associated radioactivity and release into the medium after incubation with $^{125}$I-LPS–LDL or $^{125}$I-LDL as a function of time

| Time, min | Label, μg/mg of cell protein | | | |
|---|---|---|---|---|
| | Released into medium | | Associated with cells | |
| | $^{125}$I-LPS–LDL | $^{125}$I-LDL | $^{125}$I-LPS–LDL | $^{125}$I-LDL |
| 0 | ND | * | 5.9 ± 1.3 | 0.88 ± 0.13 |
| 45 | ND | 0.24 ± 0.02 | 5.8 ± 1.8 | 0.52 ± 0.11 |
| 150 | ND | 0.51 ± 0.10 | 6.2 ± 2.0 | 0.22 ± 0.09 |
| 240 | 0.32 ± 0.19 | 0.61 ± 0.09 | 6.2 ± 1.7 | 0.16 ± 0.07 |

Human monocyte-macrophages were cultured for 7 days in 30% autologous serum and were prepared for studies as described. Each well received 0.5 ml of medium D containing 50 μg of protein per ml of $^{125}$I-LPS–LDL (202 cpm/ng of LPS) or 50 μg of protein per ml of $^{125}$I-LDL (456 cpm/ng of protein). After incubation for 3 hr at 37°C, the medium was removed, the cells were washed three times with 1 ml of medium C, and 0.5 ml of medium D was added to each well for the designated time. At each time point, the medium was removed, the cells were prepared as described for cell-associated radioactivity, and the radioactivity in the medium and that associated with the cells were determined. The values are presented as the mean ± 1 SD of sextuplicate wells. ND, not detectable.

*The amount of $^{125}$I-LDL degraded in the initial 3-hr incubation was 1.8 μg/mg of cell protein.

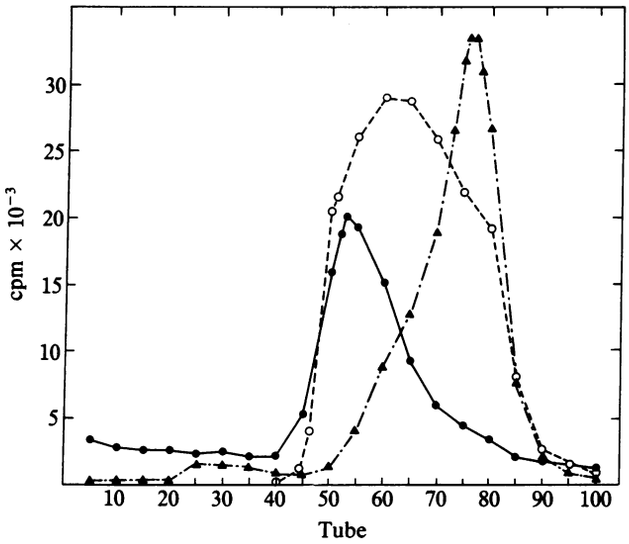


FIG. 4. Sepharose CL-2B chromatography of $^{125}$I-LDL (○), $^{125}$I-LPS (▲), and $^{125}$I-LPS–LDL complexes (●). $^{125}$I-LPS (1 mg) was injected intravenously into a WHHL rabbit, and 3 hr later the blood was withdrawn for ultracentrifugal isolation of the $^{125}$I-LPS–LDL complexes. LDL was isolated from a sibling WHHL rabbit and radioiodinated. Samples were applied and eluted as described.

**Stability of the LPS–Lipoprotein Complex.** $^{125}$I-LPS–LDL was prepared by *in vitro* incubation and was subsequently incubated with plasma, and the plasma was fractionated by ultracentrifugation. Ninety-one percent of the radioactivity remained associated with the fraction of density <1.063 g/ml. $^{125}$I-LPS–LDL prepared by *in vitro* incubation also was injected into a rabbit, and after 3 hr the plasma from the rabbit was ultracentrifugally fractionated. Eighty-nine percent of the plasma radioactivity remained associated with the fraction of density <1.063 g/ml. In other experiments $^{125}$I-LPS–LDL complexes were isolated from rabbit plasma by ultracentrifugation 3 hr after the intravenous injection of 1 mg of $^{125}$I-LPS. The $^{125}$I-LPS–LDL complexes were then incubated with normal or WHHL rabbit plasma *in vitro* for 6 hr. Upon subsequent ultracentrifugation, more than 90% of the plasma radioactivity remained associated with the LDL fraction. These experiments demonstrate that, once the $^{125}$I-LPS–LDL complexes are formed, they are remarkably stable.

## DISCUSSION

Our results demonstrate that LPS binds to the plasma lipoproteins, possibly through a lipid A-phospholipid association (20), forming an LPS–lipoprotein complex. This is a stable complex that persists despite the high salt concentrations, the high gravitational forces encountered in ultracentrifugation, and the possible effects of enzymes present in the plasma. The ultimate fate of the LPS molecule could depend upon which cholesterol-rich lipoprotein(s) predominate(s) in the plasma. Others have shown that plasma LDL binds α-toxin from *Staphylococcus aureus*, stressing the importance of LDL in the transport of toxins in the blood (21). If the LPS molecule were to bind to LDL, the LDL receptor could provide a means for the uptake of LPS by cells possessing such a receptor. In WHHL rabbits or familial hypercholesterolemic homozygotes that lack the LDL receptor, perhaps the β-VLDL receptor could potentially mediate the uptake of LPS–lipoprotein complexes.

The "response to injury" hypothesis of atherosclerosis has focused on the effects of chemical agents on cells (22). Various toxins, lipoproteins, viruses, high plasma homocystine levels, or immunological injury are among the possible factors that could lead to the development of the lipid-filled lesion (22). LPS is a naturally occurring toxin that could bind to LDL intravascularly and be transported across the endothelium as a complex, independent of the LDL receptor (23). This complex does not appear injurious to endothelial cells (5, 6). Once across the endothelium, the LPS–lipoprotein complex may be taken up by receptor-mediated endocytosis in cells such as macrophages or smooth muscle cells, or it may interact with glycosaminoglycans and remain in the artery wall (22). Recently, it has been shown that bacterial LPS from *E. coli* are retained by macrophages in spite of the degradation of the *E. coli* bacterium itself (24). The retention of LPS may have direct effects upon the cells of the arterial wall or indirect effects such as the release of factors that promote proliferation or chemotaxis. In contrast to LDL, HDL is not retained in the artery wall (25). The HDL/LDL ratio has been found to be an important predictor of atherosclerosis (26). One explanation could be that LPS bound to HDL would not be retained in the artery wall, but LPS bound to LDL or other apolipoprotein B-containing lipoproteins may be retained in the artery wall and may initiate the atherosclerotic reaction.

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